ANTI-SELF RECEPTORS

by

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Contents

A C K N O W L E D G E M E N T S

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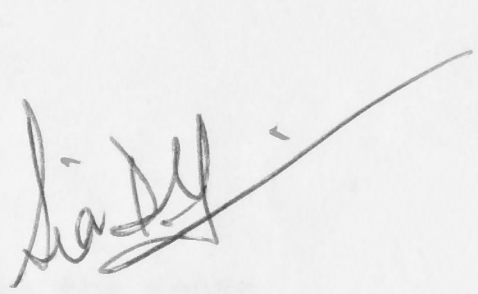
S T A T E M E N T

The purification of Autorosette Inhibition Factor (AIF) from human serum was performed in collaboration with Dr. D. B. Rylatt. The SDS-polyacrylamide gel electrophoresis of Autorosetting Receptor and Acceptor were performed by Dr. T. J. Higgins. All other experiments were my own original work and were carried out by myself under the supervision of Dr. C. R. Parish.

Part of the concluding discussion (chapter 7) has been published in a review article which was written jointly with Dr. C. R. Parish and Dr. D. B. Rylatt.



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Abbreviations Used

AIF	Autorosette-inhibition factor
B cell	Bursa of Fabricius (or mammalian equivalent)-derived lymphocyte
BCG	Bacillus-Calmette-Guerin
BSA	Bovine serum albumin
Con A	Concanavalin A
CML	Cell-mediated lympholysis
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
FCS	Foetal calf serum
G V H	Graft versus host
HBSS	Hank's balanced salt solution
H-2	Major Histocompatibility Complex in the mouse
HLA	MHC in man
HSV	Herpes Simplex virus
H-Y	Male specific antigen
Ia	I region associated
Ig	Immunoglobulin
Ir	Immune response
i.v.	Intravenous
LCM	Lymphocytic choriomenigitis virus
2 ME	2-mercaptoethanol
MEM	Eagle's minimum essential medium
MHC	Major Histocompatibility Complex

MLR	Mixed lymphocyte reaction
ml	millilitre (10^{-3} litre)
NP	(4-hydroxyl-3-nitrophenyl) acetyl hapten
PBL	Peripheral blood leucocyte
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PPD	Purified protein derivative
T cell	Thymus derived lymphocyte
T_H	Helper T cell
Tc	Cytotoxic T cell

The work described in this thesis has been the subjects of the following papers:

1. Sia, D. Y. & C. R. Parish. 1980. Anti-self receptors. I. Direct detection of H-2L region-restricted receptors on murine thymocytes. J. Exp. Med. 151: 553

2. Sia, D. Y. & C. R. Parish. 1980. Anti-self receptors. II. Demonstration of H-2L region-restricted receptors on sub-populations of peripheral T and B lymphocytes. J. Immunol. 124: 2366

3. Sia, D. Y. & C. R. Parish. 1981. Anti-self receptors. III. Lack of allelic exclusion and thymic epithelium dependence of H-2L region-restricted receptors on lymphocytes. Scand. J. Immunol. 13: 535

4. Sia, D. Y. & C. R. Parish. 1981. Anti-self receptors. IV. H-2-controlled receptors on thymocytes recognize carbohydrate structures on target cells. Immunogenetics 12: 857

5. Sia, D. Y., Rylatt, D. B. & C. R. Parish. 1982. Anti-self receptors. V. Isolation and biological properties of a serum factor that blocks autorosetting receptors on lymphocytes. Immunology 45: 207

6. Parish, C. R., Sia, D. Y. & D. B. Rylatt. 1981. Receptors for self-antigens on non-T cells. Immunology To-day 2: 172

7. Rylatt, D. B., Sia, D. Y., Mundy, J. & C. R. Parish. 1981. Auto-rosette factor: Isolation and properties of the human plasma protein. Eur. J. Biochem. 119: 641

8. Parish, C. R., Sia, D. Y. & T. J. Higgins. 1982. Automated rosetting assays for cell surface antigens and autorosetting lymphocytes. J. Immunol. Methods Submitted for publication

AIMS AND SUMMARY

The phenomenon of autorosette formation whereby lymphoid cells adhere to autologous erythrocytes has been known for several years. However, the mode of interaction and the functional significance of autorosetting has remained a mystery. The major aim of the experiments described in this thesis was to establish the molecular basis of autorosetting and determine whether the interaction is under major histocompatibility complex (MHC) control.

In chapter 3, the role of the H-2 complex in murine thymocyte autorosetting was analysed. Autorosetting was mapped precisely to the H-2L/H-2D region of the murine MHC using three experimental approaches, namely (i) inhibition of autorosetting with erythrocyte sonicates from different congenic and recombinant strains of mice; (ii) analysis of the autorosetting ability of the lymphoid cells from H-2L/H-2D mutant strains of mice; and (iii) blocking the inhibitory activity of the erythrocyte sonicates with anti-H-2 sera. This chapter also described experiments that demonstrated the autorosetting of murine peripheral T and B lymphocytes was also H-2L/H-2D controlled. In addition, B lymphocytes were shown to be the major cell type which autorosetted in the peripheral lymphoid organs, the autorosetting receptors on B cells not being immunoglobulin (Ig) or Ig-associated. Both the role of the thymic epithelium in selecting receptor specificity and the expression of autorosetting receptors on the lymphoid cells of F1 hybrid mice were also investigated. It was found that the thymic epithelium did not play a role in selecting the H-2 specificity of the autorosetting receptors, and there was no allelic exclusion in the expression of the receptors on F1 hybrid lymphocytes.

Chapter 4 described preliminary attempts to biochemically characterize the receptors and acceptors involved in autorosetting. Using sugar inhibition assays, and treating lymphoid cells and erythrocytes with proteases and exoglycosidases and then testing for their autorosetting abilities, it was concluded that the autorosetting receptor on lymphocytes was a protein and the acceptor on erythrocytes the carbohydrate portion of a glycoprotein.

Chapter 5 describes further attempts to chemically characterize the receptors and acceptors mediating autorosetting. A procedure was devised for extracting from murine thymocytes an agglutinin of mouse thymocytes that appeared to represent the autorosetting receptor(s). This conclusion was based on (i) the observation that the same sugar inhibited the haemagglutinin as blocked autorosetting, (ii) genetic analysis of sugar inhibition patterns that suggested the haemagglutinin was under H-2L/H-2D region control, (iii) the detection of substantially less haemagglutinin in thymocyte lysates of the H-2L deletion mutant mouse strain dm2, a strain that lacks autorosetting receptors and (iv) the ability of purified autorosette inhibition factor (AIF) (see chapter 6) to inhibit the haemagglutinin. This chapter also describes absorption and sugar elution procedures for isolating autorosetting receptors from thymocytes and acceptor molecules from erythrocytes. By running ^{125}I-labelled material on SDS-PAGE the thymocyte receptor was found to have a mol. wt. of 63,000 daltons and possibly consisted of two subunits whereas the erythrocyte acceptor molecule was a single polypeptide of mol. wt. 62,000 daltons.

In chapter 6, the biological properties of an autorosette inhibition factor (AIF) in normal serum were investigated. Blocking experiments revealed that the inhibitory factor was neither

strain nor species specific. Furthermore, AIF appeared to be secreted by a comparatively radiosensitive macrophage or monocyte as *in vivo* administration of carrageenan or silica markedly depressed AIF levels in serum and *in vitro* studies demonstrated that AIF was secreted by adherent peritoneal cells. Finally, a one step procedure for isolating pure AIF from human serum was devised. The factor was shown to be a single polypeptide chain of mol. wt. 81,000 daltons and was present in human serum at a concentration of 50-100 µg/ml.

Finally, in chapter 7, the general significance of the experimental results presented in this thesis, and the theoretical implication of the finding that autorosetting representing a MHC-controlled carbohydrate-protein interaction between cells are discussed.

Whatever you do,

do it well.

My father

To my Father and Mother .....

Chapter 1

General Introduction

Table of Contents

1. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) OF THE MOUSE

1.1 Historical Background

The original discovery of the Major Histocompatibility Complex (MHC) was made by Gorer (1936) in his analysis of blood group antigens in mice. In an attempt to identify the blood group antigens in inbred mouse strains, he immunised rabbits with erythrocytes from different strains of mice. When the resulting antisera were tested for their abilities to agglutinate red cells of these inbred mouse strains, Gorer was able to identify four different red cell antigens. One of these antigens, denoted as antigen II, was found to be present in some mouse strains and inherited in a simple Mendelian manner. In addition, the antigen II negative F1 mice derived from breeding between antigen II positive and antigen II negative parents were found to be able to reject tumour grafts derived from antigen II positive strains. In contrast, antigen II positive F1 mice failed to reject a tumour from antigen II positive parents (Gorer 1937). For this reason, Gorer designated antigen II a histocompatibility antigen.

In a joint study over the next few years, Gorer, Lyman and Snell (1948) showed that the gene(s) that code for antigen II mapped to chromosome 17. Antigen II and its controlling gene(s) were then named histocompatibility-2-hence abbreviated as H-2. Subsequent work carried out by Snell and co-workers using inbred mouse strains to analyse H-2 further demonstrated that it did not consist of II^+ and II^- alleles alone, but a series of closely linked loci. These loci were unique in that they controlled separate transplantation antigens. These findings led to the concept of the Major Histocompatibility Complex, or MHC.

1.2 Genetic Map of the H-2 Complex

The H-2 complex is located on the IX linkage group of the murine 17th chromosome, is 0.3 - 1.5 cM long and contains 10 - 15 known loci. Linked to the H-2 are the minor histocompatibility loci (H-31, H-32, H-33 and H-39), loci coding for various isoenzymes (Pgk-2, Ce-2, Ap-1, Glo-1, Map-2), an immune response locus (Ir-5) and blood group locus (Ea-2), and a T complex which is known to affect embryonic differentiation, chromosome segregation, and sperm behaviour (Klein and Hammerling, 1977). The current map of the H-2 complex is shown in Fig. 1.1. The complex can be divided into at least six regions (K, I, S, U, D and T), with the I region further divided into six subregions (N, A, B, J, E and C). With the exception of the S region, the gene products of all these regions are expressed on cell surfaces. Although the T region is frequently presented in maps of the H-2 complex, its function is obscure and will not be discussed further.

Since its original definition by Gorer the H-2 complex has been shown to control a wide range of immunological and non-immunological phenomena. Thus, at the immunological level, the H-2 complex controls transplantation antigens, lymphocyte activating determinants (Lad) involved in mixed lymphocyte reactions (MLR) and graft-versus-host reactions (GVHR), target antigens for cell-mediated lympholysis (CML), alloantigens of restricted cellular distribution and immune responsiveness to various antigens (Ir genes). A summary of the various genes in the H-2 complex that control these different immunological effects is presented in Table 1.1.

On the other hand, numerous non-immunological effects are controlled by the MHC, such as cyclic AMP levels in the liver, testosterone levels, cortisone-induced cleft palate and mating

Figure 1.1

Genetic Map of the Murine MHC (Modified from Klein 1981)

Regions	K	I						S	U	D	T
Subregions		N	A	B	J	E	C				
Gene Products	H-2K	Ia	Aα Aβ Eβ	?	Ia	Eα	Ia	Ss Slp	H-2U	H-2D H-2L H-2R H-2M	Tla

Centromere ← → Telomere

Fig. 1.1 A genetic map of the MHC complex of the mouse (H-2 gene complex)

The gene products of the loci designed are as follows: H: Histocompatibility antigens; Ia: I-region-associated antigens; Ss: Serum serological; Slp: sex-linked protein; Tla: Thymus-leukaemia antigen

Table 1.1

The Mapping of Different Genes and Functions within the H-2 Complex

Traits associated with H-2	H-2 regions and subregions						
	H-2K	I-A	I-B	I-J	I-E	I-C	H-2D
Transplantation antigens	+	+	-	?	?	+	+
Lad (MLR, GVHR)	+	+	-	+	+	+	+
CML determinants	+	+	?	?	?	?	+
H-2 (class I) antigens	+	-	-	-	-	-	+
Ia (class II) antigens	-	+	-	-	+	+	-
Ia on B cells	-	+	-	-	+	+	-
Ia on T cells	-	+	-	+	+	+	-
Ir genes	?	+	+	?	(+)[a]	+	?

a As Ir genes were initially mapped to the I-C subregion which also included I-E the precise mapping of these Ir genes is uncertain.

preference. In fact, almost 60 different traits have been found to be associated with H-2 (Klein, 1978). These observation have generated great interest in the H-2 complex and, in particular, have stimulated researchers to determine the role played by the serologically defined antigens in these phenomena.

1.3 The K and D Regions

The gene products of these regions were originally described by Gorer (1936) as blood group antigens. The antigens of these regions are known to play an important role in the rejection of transplantable tumours and normal tissues (Gorer 1937) (Table 1.1).

The H-2K and H-2D antigens are expressed on virtually all tissues and exhibit a number of distinct antigenic determinants or "specificities". These specificities can be detected serologically by conventional or monoclonal antibodies, and they can be assigned as public or private specificities. Public specificities are defined as those shared by two or more alleles and they are carried by the H-2K and H-2D molecules of different inbred mouse strains or of different H-2 haplotypes. Private specificities, on the other hand, are unique antigenic determinants restricted to a single allele and thus are found on the H-2K or H-2D molecules of a particular inbred mouse strain. To date, over 100 public and private specificities have been detected on the H-2K and H-2D antigens.

The direct evidence for K/D polymorphism has come from studies of wild mouse populations (Klein and Zaleska-Rutcznska 1977; Wakeland and Klein 1979a, b; Duncan and Klein 1980). Most of the wild mice were found to be heterozygous at the K and D loci. The phenotypic frequencies of the public specificities of the H-2K and H-2D antigens were much higher than those of their private specificities (0.5 compared to 0.01 - 0.05). Based on these frequencies and other

relevant considerations, at least 100 alleles could be estimated at the K or D locus of wild mice. The high degree of polymorphism of the K or D locus has been suggested to reflect varying selective pressure on the wild mouse population (Klein 1980).

Despite the clear distinction between the H-2K and H-2D regions, based on genetic, capping and sequential immunoprecipitation studies (Cullen et al.1972; Neauport-Sautes et al. 1973) there are striking similarities in the properties of the H-2K and H-2D molecules. Firstly, some public H-2 antigenic specificities are expressed on both the H-2K and H-2D molecules (Shreffler et al. 1971). Second, both molecules are glycoproteins with a very similar molecular structure (see section 1.3.2) and, thirdly, peptide mapping and limited amino-acid sequence data have demonstrated considerable sequence homology between the two molecules (Vitella and Capra 1978). These findings clearly support the postulate that these two loci arose by gene duplication, a point which will be discussed later (see section 1.3.2).

Although the H-2K/D regions were originally described as single loci, recent evidence has demonstrated a greater genetic complexity in these regions, new loci associated with both H-2K and H-2D being identified (Fig. 1.1). The most clearly established locus is H-2L, which is associated with the H-2D region and will be discussed in detail below (section 1.4). However, in addition to this locus, recent studies using cocapping techniques have identified another serologically distinct $H\text{-}2D^{d}$ region associated antigen which has been designated H-2M (Ivanyi and Démant 1979). Futhermore, serological analysis of D^{k} region products with antisera to public and private specificities has revealed the existence of two separate molecules, designated $H\text{-}2D1^{k}$ and $H\text{-}2D2^{k}$ (Démant et al. 1981). Finally, Hansen et al.

(1981) have identified another molecule under D-region control, termed H-2R, which could be serologically distinguished from H-2L. However, it is uncertain whether this molecule is coded for by a third H-2D locus or merely represents a precursor or conformational variant of the D or L molecule.

Monoclonal anti-H-2 antibodies have also revealed genetic complexity in the K region. O'Neill et al. (1981) reported heterogeneity in K^k molecules, as the antigens detected by different monoclonal antibodies redistributed independently on the cell surface and differed in their chemical properties, one being a protein and the other a glycolipid molecule. Support for K region heterogeneity was reported by Eskinazi et al.(1981) who found two H-2K molecules that had a differential expression on subpopulations of spleen cells and could be separated by sequential immunoprecipitation. In addition, a new genetic region designated H-2U, that maps between the S and D regions has been recently defined by O'Neill and Parish (1981). Serological and biochemical studies revealed that the antigens of this region resembled Ia antigens in that they were expressed predominantly on B cells, and were made up of two different molecular weight structures, one of 36,000 and the other 60,000 daltons resembling the dimeric characteristics of Ia antigens.

1.3.1 Cellular Distribution of H-2K and H-2D Antigens

The H-2K and H-2D antigens are expressed in most tissues or organs. The plasma membranes of cells of the immune system, such as lymphocytes and macrophages, as well as liver cells are particularly rich in these molecules. Most somatic cells such as muscle cells, fibroblasts, red cells and nerve cells, on the other hand, have low contents of these antigens. Very low amounts of H-2K and H-2D molecules

are also detected on the membranes of early differentiating cells such as germ cells and early embryos (Klein 1975). Recently, O'Neill and Blanden (1979) have reported that the quantitative expression of H-2K and H-2D antigens on lymphoid cells may have immunological significance. Thus, the expression of lower amounts of H-2K and H-2D antigens on the spleen cells is paralleled by the lower efficiency of the F1 cells both to be recognized as target and to act as stimulators for cytotoxic T cells against the parental H-2K and H-2D antigens.

1.3.2 Molecular Structure of the H-2K and H-2D Antigens

Chemical studies have revealed that the H-2K and H-2D antigens are glycoprotein molecules of molecualr weight 45,000 daltons (Silver and Hood 1976; Cullen *et al*. 1976). This glycoprotein molecule is made up of a polypeptide chain of molecular weight 39,000 daltons with two carbohydrate units each of 3,000 daltons covalently attached to it. Noncovalently associated with this polypeptide is β2-microglobulin of molecular weight 12,000 daltons which is coded for by the non-MHC linked genes and has a striking sequence homology with the heavy chain constant region of immunoglobulins.

The antigenic determinants of the H-2K and H-2D antigens appear to be determined by variations in the amino-acid sequence of the 45,000 dalton chain (Cullen *et al*. 1976; Vitetta and Capra 1978). However, recent studies with monoclonal antibodies suggest that some antigenic determinants are dependent upon the 45,000 dalton chain being associated with β2-microglobulin (I. F. C. McKenzie, personal communication). Although there is no evidence that the carbohydrate portion of the H-2 glycoproteins are antigenic, it was recently reported that the K-region may control a second class of H-2 antigens that are carbohydrate in nature and are expressed as

glycolipids on cell surface (O'Neill et al. 1981). These glycolipid H-2 antigens appear analogous to the carbohydrate-defined Ia antigens discussed later (section 1.5.6).

The large polypeptide chain of the H-2 molecule contains four-half cysteines which combine to form two disulphide bridges; each bridge forming a loop with a molecular weight of approx. 12,000 daltons. The possible structure of the H-2K and H-2D antigens is depicted in Fig. 1.2. The 45,000 dalton glycoprotein can be divided into four regions. The first region is a hydrophilic amino-terminal region consisting of a 8,000 dalton protein moiety with one of the carbohydrate chain covalently attached to it. The second region corresponds to the 12,000 dalton fragment containing the first cysteine disulphide loop (C1) and has the second carbohydrate chain covalently attached to it. With its presumed sequence homology to immunoglobulin, it is proposed that this C1 fragment could non-covalently associate with β2-microglobulin. The third region is very similar to the second region and has the second cysteine disulphide loop (C2). The fourth region is hydrophobic; half of its 8,000 dalton fragment is transmembrane, the other half being intracellular. The intra-cellular segment is believed to served as a mediator of interactions with the cytoplasm (Silver and Hood 1976).

Direct amino acid sequencing studies of the H-2K and H-2D gene products of different mouse strains revealed that allelic products of either gene display approximately the same degree of homology among themselves. For instance, K^k versus K^b, and D^k versus D^b molecules were found to differ at some 40 - 50 amino acids. The K and D antigens of the same H-2 haplotype e.g., (K^b, D^b; K^k, D^k; etc.), on the other hand, were found to differ at about the same number of positions (Hehning et al. 1976; Evenstein et al. 1976, 1978; Coligan et

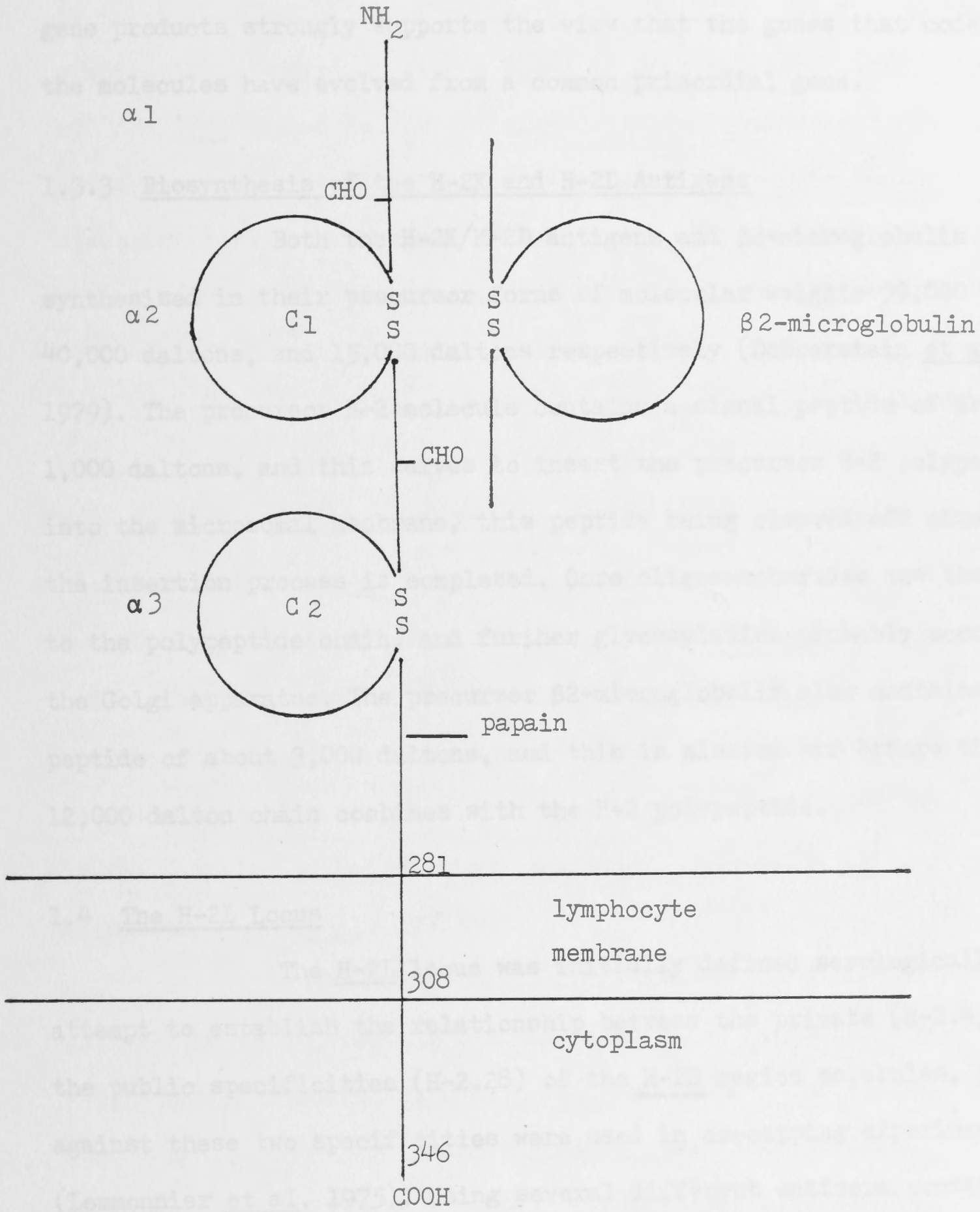

Fig. 1.2 A schematic representation of the murine membrane-bound H-2K (D) molecule as deduced from different analysis. The molecule is comprised of four domains: α1, α2 and α3, while β2-microglobulin forms the 4th domain.

The numbers designate the amino acid positions. S S: cys-cys bonds; CHO: carbohydrate; C1: first cysteine disulphide loop; C2: second cysteine disulphide loop. (adapted from Klein 1979; Owen 1981)

al. 1978; Maizels et al. 1978). The β2-microglobulin polypeptide showed significant homology with the constant region of certain immunoglobulin molecules (Poulik 1975). The sequence homology of the H-2K and H-2D gene products strongly supports the view that the genes that code for the molecules have evolved from a common primordial gene.

1.3.3 Biosynthesis of the H-2K and H-2D Antigens

Both the H-2K/H-2D antigens and β2-microglobulin are synthesized in their precursor forms of molecular weights 39,000 - 40,000 daltons, and 15,000 daltons respectively (Dobberstein et al. 1979). The precursor H-2 molecule contains a signal peptide of about 1,000 daltons, and this serves to insert the precursor H-2 polypeptide into the microsomal membrane, this peptide being cleaved off after the insertion process is completed. Core oligosaccharides are then added to the polypeptide chain, and further glycosylation probably occurs in the Golgi apparatus. The precursor β2-microglobulin also contains a signal peptide of about 3,000 daltons, and this is cleaved off before the 12,000 dalton chain combines with the H-2 polypeptide.

1.4 The H-2L Locus

The H-2L locus was initially defined serologically. In an attempt to establish the relationship between the private (H-2.4) and the public specificities (H-2.28) of the H-2D region molecules, antisera against these two specificities were used in co-capping experiments (Lemmonnier et al. 1975). Using several different antisera containing antibodies against the H-2.28 family of specificities it was found that not all the molecules with these public specificities co-capped with molecules bearing the private specificities (H-2.4). These findings, therefore, implied that in addition to the H-2D region-associated molecules

bearing private and public specificities, another category of H-2D-region molecules existed that only carried public specificities. Subsequently, this finding was confirmed by Hansen et al. (1977) by immuno-precipitation of murine spleen cell lysates with D-region specific allo-antisera. It was found that an antiserum which removed all the H-2D region molecules carrying the private specificity did not remove all molecules reactive with an antiserum to the public specificities. The existence of a new gene product coded by the D-region was further supported by peptide mapping studies that revealed peptide differences in the molecule recognized by anti-H-2.28 and anti-H-2.4 sera although these molecules had similar molecular weights and were associated with β2-microglobulin (Demant and Neauport-Sautes 1978). Support for the new locus was also gained from studies with the H-2^{d} 'loss' mutant BALB-H-2^{dm2} in which (i) a loss (deletion) of some H-2.28 was detected, whereas the H-2.4 molecule remained intact, and (ii) antisera detecting the 'H-2.28 family' of antigens could be raised between mutant and parent (McKenzie et al. 1977; Morgan et al. 1978). In accordance with the standing rules of H-2 nomenclature, the new locus in the D-region of H-2 was then named H-2L (Demant et al. 1977; Neauport-Sautes et al. 1978)

Subsequent serological studies have shown that the anti-H-2.28 sera detecting H-2L in fact detect a family of specificities: H-2.28 which is shared between H-2K, H-2D anf H-2L molecules, and H-2.64 and H-2.65 that are only found on the H-2L molecule (Demant et al. 1978; Neauport-Sautes et al 1978). Furthermore, a serological examination of twenty-one D-end recombinant mouse strains for their H-2L specificities revealed that H-2L antigens correlated with H-2D antigens, thus implying that the H-2D and H-2L loci are tightly linked (Hüang et al. 1979). This study also demonstrated a high polymorphism for the

H-2L locus, and it is possible that this will be increased further when more anti-H-2L antisera become available.

The cell surface expression of H-2L molecules has recently been examined by Potter et al. (1981). By employing monoclonal anti-H-2L antibodies in a flow microflurometry analysis, it was found that the cellular distribution of H-2L molecules was the same as that of the H-2K and H-2D antigens. However, there was about 2 - 3 fold less H-2L molecules expressed on lymphocytes than H-2K and H-2D antigens. In addition, as with the H-2K and H-2D molecules, the expression of H-2L antigens on F1 lymphoid cells did not show allelic exclusion.

The primary structure of the H-2L molecule has been compared to that of H-2K and H-2D gene products in different mouse strains. Peptide maps of H-2L^{d} antigen immuno-precipitated from H-2^{d} tumour cells by specific allo-antisera have revealed amino acid sequence homology with the H-2K^{b} and H-2D^{d} gene products (Coligan et al. 1979). On the other hand, the differences between the H-2L^{d} molecule and these other transplantation antigens appear to be distributed throughout their polypeptide chains. Thus the primary structure of the H-2L^{d} molecule substantiates its classification as a classical transplantation antigen, but indicates it is distinct from the H-2K and H-2D molecules.

Analysis of the tryptic peptide maps of the H-2L^{q} and H-2L^{d} molecules isolated from spleen cells has revealed remarkable similarities (Rose et al. 1980). On the other hand, some differences in amino acid sequence between the two molecules were observed, despite no serological differences being detected between these molecules. These findings imply that the serological determinants of these molecules were not affected by the changes in sequence, although whether T cells can recognize such changes remains to be

determined.

At the functional level, studies of the H-2^{dm2} mutant, which lacks H-2L, have shown that the H-2L molecules can act as strong transplantation antigens, stimulator antigens for mixed lymphocyte cultures and target antigens for alloreactive cytotoxic T (Tc) cells (Melvold and Kohn 1976; McKenzie et al. 1977a, b; Hansen and Levy 1978). Furthermore, in studies of the H-2-restricted Tc response to ectromelia virus-infected cells, Blanden and co-workers (Blanden et al. 1977; Blanden and Kees 1978) have shown that for D-region restricted lysis, the H-2L molecule, which is lost in the mutant, is required for the induction of Tc to virus-infected cells but is not necessary for the lysis of targets. On the other hand, blocking studies with anti-H-2L antibodies have demonstrated that some influenza virus-specific Tc probably recognize virus plus H-2L (Biddison et al. 1978). The H-2L molecule therefore appears to be able to act as a restriction element in both the inductive and effective phases of the Tc responses, as can the H-2K and H-2D molecules.

1.5 The I Region

The central region of the H-2 complex (I-region), which codes for the Ia antigen, was originally defined as result of the analysis of H-2 recombinant mice for their antibody responses to synthetic polypeptide antigens (Shreffler and David 1975; Klein 1975). Subsequently, an immune response gene, designated Ir-1, occupying a chromosome segment between the H-2K and S regions (i.e., the I (immune response)region) was shown to genetically control and primarily determine the responsiveness to these antigens. Further analysis of the ability of various inbred and congenic mouse strains to respond (i.e., high responsiveness as opposed to low or non-responsiveness) to many

different synthetic polypeptide antigens led to the subdivision of the I-region into two subregions, Ir-IA (originally termed Ir-1) and Ir-IB.

With the use of H-2 recombinant mice a greater genetic complexity in the I-region has become apparent. The I-region is currently subdivided into six subregions namely N, A, B, J, E and C arranged in this order from the centromeric to the telomeric ends. Each of these subregions may contain one or more loci controlling different traits such as serologically detectable antigens (Ia), immune response (Ir) genes, lymphocyte activating determinants (Lad) and histocompatibility antigens (H) (Table 1.1).

1.5.1 The I-N Subregion

The I-N subregion was recently identified by Hayes and Bach (1980). By absorbing an (ATL x B10.D2)F1 anti-B10.BR antiserum with B10.T(6R) lymphocytes, the antiserum was still found to retain activity on AQR lymphocytes. Based on this observation and additional functional studies the I-N subregion has been proposed to exist between the K region and the I-A subregion.

1.5.2 The I-A and I-E Subregions

1.5.2.1 Cellular Distribution

The I-A subregion contains the Ia-1 locus which codes for Ia specificities which are predominantly found on B cells. In addition, Ia antigens encoded by the I-A subregion are expressed on epidermal cells, Langerhan cells in the skin (Frelinger et al. 1978), splenic macrophages (Cowing et al. 1978), T cell blasts, Fc receptor (FcR^+) T cells (Stout et al. 1977) and cytotoxic T cells against some syngeneic tumours (Fujimoto et al. 1978). Allogeneic

effect factor (AEF) which acts on B cells has also been reported to bear I-A subregion coded determinants (Armerding et al. 1974). So far, I-A subregion encoded antigens have not been reproducibly detected on helper and suppressor T cells (Murphy et al. 1976). Serological analysis has so far revealed that the Ia specificities detected on B cells are distinct from those on T cells, or T cell derived factors (Tohyhisa et al. 1978; Oi et al. 1978; Hammerling et al. 1979). The Ia-1 B specificities like the H-2K and H-2D region coded specificities, can also be classified as public and private. For example, Ia.2 is the 'private' Ia^k specificity whereas Ia.1, 3, 5 and 18 are public specificities (Shreffler and David 1975). To date, over 40 Ia specificities within the I-A/I-E subregions have been defined serologically (Shreffler and David 1975; Müller 1976). The I-A subregion also contains a histocompatibility locus, H-2IA (Klein et al. 1976).

The I-E subregion was originally defined by the detection of the Ia.22 specificity encoded by the Ia-5 locus present in the $H\text{-}2^k$ haplotype (Shreffler et al. 1977). Recently, Ia.23 specificity of $H\text{-}2^d$ and Ia.7 which is a public specificity of $H\text{-}2^k$ and $H\text{-}2^d$ have also been mapped to the I-E subregion (Okuda and David 1978; Murphy et al. 1976). These specificities are predominantly present on B cells. Co-precipitation studies also have shown that they are present on one Ia molecule, thus implying that they are all controlled by one locus in the I-E subregion (Shreffler et al. 1977; David and Cullen 1978; Cullen et al.1980). Other cell types which express these Ia specificites include a proportion of splenic macrophages (Cowing 1978) and epidermal cells (Frelinger 1978). Also, I-E specificites reacting with antisera raised against Con-A induced T cell blasts have been demonstrated on T cells, but not on B cells, thus suggesting that a new locus exists within the I-E subregion (Hayes and Bach 1978).

The I-E subregion also contains a Lad locus, but it is not clear whether this is identical with the Ia-5 locus (Peek et al. 1977; Okuda and David 1978).

1.5.2.2 Molecular Structure of the I-A and I-E Subregion Gene Products

Based on the potential importance of the I-A and I-E subregion antigens in immuno-regulation, work has been stimulated towards the serological and biochemical characterization of these gene products.

Like the isolation of the H-2K and H-2D antigens, murine Ia antigens coded for by the I-A and I-E subregions on B lymphocytes are usually obtained from detergent extracts of radio-labelled lymphocytes by immuno-precipitation with specific allo-antisera. Immuno-precipitates of these antigens from both these subregions usually yield molecules ranging in molecular weights from 57,000 - 63,000 daltons (Schwartz and Cullen 1978) that are dissociated into two-identical polypeptide chains, termed α and β, one of about 33,000 daltons, and the other of 28,000 daltons. These α and β polypeptides are glycoproteins with the carbohydrate moiety constituting some 10 - 15% of each chain (Freed and Nathenson 1977). In some cases, the α and β polypeptides can be precipitated independently by monospecific anti-Ia antibodies, thus implying they are likely to be noncovalently associated. The antigenic activity of these glycoprotein molecules appears to reside in their protein portion (Cullen et al. 1975), but there is evidence for a second family of Ia antigens that are carbohydrate in nature (see section 1.5.7). It is not clear at present which specificities are associated with the α and β chains of the molecule. Recently, Mckean et al. (1981) have detected an alteration in the β chain of the Ia molecule in the I-A subregion mutant

B6.C-H-2^{bm12}. However, it may also be that an intact α - β complex is required for the detection of the Ia specificities.

Biochemical analysis has indicated that the I-E subregion products are similar to the Ia-1 locus products, having an α (33,000 daltons) and a β (28,000 daltons) chain structure (Schwartz and Cullen 1978). Subsequent studies by Jones et al. (1977, 1978, 1978b) using two dimensional gel electrophoresis analysis have revealed that I-A and I-E subregion molecules are three chain structures composed of two highly polymorphic chains (α and β), and one non-polymorphic chain, the Ia associated invariant chain (Ii) of molecular weight about 31,000 daltons. A point of particular interest arising from these studies is that one chain (β) of the I-E molecule is coded for by the I-A subregion, whereas the other (α) is coded for by the I-E subregion (Jones et al. 1978). The α and β chains of the I-A antigens are, however, both encoded by the I-A subregion. In addition, the I-E coded product appears to regulate the cell surface expression of the I-A subregion product (David and Cullen 1978). More recently, it has also been shown that an Ia locus mapping in I-A/I-J regulates the quantitative expression of the I-E subregion controlled α chain (Murphy et al. 1980). Analysis of the Ia antigen immuno-precipitated from tunicamycin-treated murine spleen cells has so far revealed that Ii contains two N-linked carbohydrate units (Sung and Jones 1981). So far, Ii has been found to be absent from Ia preparations from plasma membranes. Thus Ii seems to associate specifically with the α and β chains inside the cells, and is unlikely to be a structural component of the Ia antigens on plasma membranes.

Genetic and biochemical studies have revealed that Ia antigens may vary diversely in their structures and expression on cell membranes of different mouse strains. Many mouse strains express

two distinct complexes of Ia antigens, Aα:Aβ and Aβ:Eα on their cell membranes. Cells from F1 animals can express mixed haplotype Aα:Aβ and Aβ:Eα complexes in addition to the parental forms (Jones et al. 1981; McNicholas et al. 1982). As a result, individual cells from an I-region heterozygote can express as many as eight different sets of Ia complexes.

The significance of these findings lie not only in the fact that these interactions between different I-subregion products can generate 'new' Ia antigneic specificities, but also as they may provide the basis for an understanding of the dual Ir gene control of immune responses, e.g., Ir gene complementation in F1 hybrids between non-responder parents. At this point it should be noted that although complementing Ir genes were initially mapped to I-A and I-C subregions (Benacerraf and Germain 1978), the I-C Ir genes could be in the I-E subregion, since the original I-C subregion contained both I-E and I-C.

Peptide mapping and amino-acid sequencing studies of the N-terminal ends of the murine Ia antigens revealed that the α and β chains of the I-A or I-E subregion molecules are not homologous to each other or to the H-2K and H-2D antigens (Silver et al. 1977; Freed et al. 1978; Cook et al. 1979a, b). Considerable homology, however, has been found among the α chain of various mouse haplotypes. On the other hand, substantial sequence variations were seen among the β chains, thus suggesting that the β chain is predominantly involved in defining the serological Ia specificities.

Studies on wild mice so far have revealed that I-A subregion polymorphism is high-approaching close to that of the K and D loci (Wakeland and Klein 1979b). Another degree of genetic polymorphism is also revealed in the primary structure of the Ia polypeptide chains,

namely Aα, Aβ and Aβ, Eα ; in addition, as discussed above, they combine in diverse forms to form different Ia complexes (Cook et al. 1979; McMillan et al. 1981). The I-E subregion is less polymorphic. The number of I-E alleles has been estimated to be about 10 (Klein 1981).

1.5.3 The I-B Subregion

The I-B subregion was originally defined by the presence of Ir genes which control the response to an IgG myeloma protein (Lieberman and Humphrey 1971). In addition, Ir genes regulating responsiveness to other antigens, such as lactate dehydrogenase B (Melchers et al. 1973),and Staphylococcal nuclease (Lozner et al. 1974; Sachs et al 1977) were also mapped to the I-B subregion. So far, no Ia antigens associated with I-B subregion have been defined, and in fact the existence of the the I-B subregion has been questioned (Benacerraf and Germain 1978). However, an Ir gene controlling responsiveness to the male specific H-Y antigen for the delayed-type hypersensitivity reaction (DTH) and graft rejection, but not for the Tc response to H-Y has recently been mapped to the I-B subregion (Hurme et al. 1978; Liew and Simpson 1980).

1.5.4 The I-J Subregion

The I-J subregion is marked by the Ia-4 locus. It was originally defined by functional criteria, where allo-antisera, by cytotoxicity, were shown to remove T cells which suppress the production of immunoglobulin of the Ig-1 allotype (Murphy et al. 1976). The I-J subregion antigenic determinants have also been detected on other suppressor T cells, including carrier specific, idiotype specific and tumour specific suppressor cells (Murphy 1979). Besides being present on suppressor cells, I-J subregion antigenic determinants are also detectable on: a) cells bearing the acceptor sites for suppressor

factors (Tada et al. 1977); b) T cell derived antigen specific suppressor factors (Tsf), which are involved in suppressing both humoral (Tada et al 1976) and cellular immunity (Greene et al 1977); c) a macrophage subpopulation required in the generation of a primary in vitro antibody response (this reaction is only blocked by anti-I-J serum (Niederhuber and Allen 1980); d) Con A promotor T cells required for responsiveness of $I\text{-}J^-$ T cells to Con A (Frelinger 1977); e) T cells which stimulate in the MLR (anti-I-J serum blocks this reaction) (Okuda et al 1977). Whether these determinants are coded for by the Ia-4 locus, or are encoded by another locus in the I-J subregion is still unclear. There is a recent report suggesting that the I-J determinants expressed on accessory cells (non-T/non-B) are different from those found on suppressor T cells (Murphy et al. 1981).

In more recent studies, I-J subregion determinants have been detected on other immuno-regulatory T cells. Thus, at least two $I\text{-}J^+$ T cell subsets have been reported to operate in the generation of suppressor inhibitory activity, referred to as contrasuppression . The first subset, the inducer of contrasuppression, secrets an $I\text{-}J^+$ soluble mediator which acts on the second subset (i.e., the contrasuppressor cells) which in turn inhibits suppressor T cell effector function (Gershon et al. 1981). Whether the I-J determinants expressed on these two $I\text{-}J^+$ subsets of T cells are distinct from those detected on other cell types is still unclear.

Studies from Tada et al. (1978b) have demonstrated the presence of another locus in the I-J subregion, which codes for determinants on a subpopulation of helper T cells (Th2). By cross-absorption analysis of an anti-I-J serum it was shown that the I-J determinants of $I\text{-}J^+$ helper T cells (Th2) were distinct from those expressed on suppressor T cells.

In contrast to the I-A and I-E subregion coded antigens, the I-J$^+$ cells have been hard to detect, only 8-10% of normal spleen T cells being found to be I-J-positive as detected by a rosetting technique (Parish and McKenzie 1977), while following enrichment for antigen-binding T cells 30% I-J$^+$ T cells could be detected by cytotoxicity (Okumura et al. 1977). So far, I-J antigens have not been detected on B cells.

The polymorphism of the I-J subregion is unclear, and so far there have been no chemical studies on I-J subregion products.

1.5.5 The I-C Subregion

The I-C subregion is defined by the Ia.6 specificity, which is only found in two haplotypes (d,p) (Shreffler and David 1975). The antisera which led to the original definition of this subregion contained an IgM antibody that was only found in early bleeds of the immunised mice (David 1976). Since then, there have been difficulties in producing anti-Ia.6 antibody, and hence demonstrating the existence of the I-C subregion. The I-C and I-E subregions have, therefore, been considered for some time by many investigators as a single subregion, I-E/I-C (Murphy 1980). Recently, Sandrin et al. (1981) have been able to make an antiserum which detects the Ia.6 specificity and hence have confirmed I-C as a distinct subregion in the H-2 complex.

Based on the reactivity of antisera to the Ia.6 specificity, I-C subregion coded determinants have been detected on T cell blasts and FcR$^+$ T cells (Frelinger et al. 1976; Stout et al. 1977). So far, the Ia.6 specificity has been reported to be absent from B cells (David et al. 1976; McKenzie et al. 1980). On the other hand, using xenogeneic antisera, carbohydrate-defined Ia specificities controlled by a gene

within the I-C subregion have been detected, these antigens being predominantly expressed on B cells (McKenzie et al.1980). This result, therefore, implies that there is a second locus in the I-C. Also encoded within I-C are lymphocyte activating determinants expressed on a suppressor factor generated in a MLR (Rich et al. 1979), and transplantation antigens (McKenzie and Henning 1976).

1.5.6 Carbohydrate-defined Ia Antigens

The cell surface Ia alloantigens described in the previous sections have been clearly shown by biochemical analysis to be glycoprotein molecules whose antigenic determinants reside in their protein portion (Schwartz and Cullen 1978). In contrast, a second class of Ia antigens have been described whose antigenic determinants appear to be carbohydrate in nature. These Ia antigens were initially detected in mouse serum, as shown by the ability of mouse serum to specifically inhibit the binding of cytotoxic anti-Ia sera to spleen cells (Parish et al. 1976). This second class of Ia antigens can be distinguished from the classical glycoprotein Ia antigens, as they appear to be glycosphingolipid molecules of low molecular weight, i.e., 2000-5000 daltons (Parish and McKenzie 1980). Further evidence for these antigens comes from the ability of sugars to inhibit the binding of certain glycosidases, such as neuraminidase, expose Ia antigens of foreign haplotype on cells (Parish et al. 1981) and from the characterisation of monoclonal anti-Ia antibodies that recognize carbohydrate determinants (Higgins et al. 1980). A particularly interesting aspect of the monoclonal antibody studies is that some of the specificities detected by the monoclonals were indistinguishable on the basis of genetic analysis (and, therefore, have been assigned the same numerical specificity), yet differed

in chemical composition, i.e., carbohydrate-defined and protein-defined Ia.2 and Ia.17 specificities were detected.

The two systems of Ia antigens, although chemically different, have several features in common. Both systems are highly polymorphic, have a similar cellular distribution and are controlled by genes in the I-A, I-E and I-C subregions of the H-2 complex (Parish and McKenzie 1980). On the other hand, these two systems of antigens do differ in their biological properties. For example, the carbohydrate-defined Ia antigens are found in high concentrations in serum and tissue fluids whereas the protein-defined Ia antigens are not. Furthermore, these soluble Ia antigens are secreted by Ly-1^{+}2^{-} T cells, the rate of secretion increasing rapidly during certain immune responses (Parish and McKenzie 1980). Recently, it has been shown that carbohydrate Ia antigens are carried on certain immuno-regulatory factors.

It should be noted, however, that in spite of the evidence for the presence of Ia glycolipid molecules, there has been some controversy as to the existence of these molecules (Schwartz and Cullen 1978). These studies, therefore, clearly require confirmation in other laboratories.

1.6 The S Region

The S region, situated in the centre of the H-2 complex, was originally defined by variations in the levels of a serologically detectable serum protein, termed Ss (Shreffler and Owen 1963), and Slp, a sex linked variant of this protein (Passmore and Shreffler 1970). Subsequently, the Ss protein was identified as the fourth component of complement (C4) (Lachmann et al., 1975; Curman et al., 1975; Meo et al., 1975).

The Ss locus codes for a 185,000 dalton single chain molecule, which is post-transcriptionally split into the α, β and γ chains (Roos et al. 1978). The Slp locus apparently is distinct from the Ss locus. The Slp protein lacks C4 activity, and its function is unknown (Ferreira et al. 1978).

2. H-2 Restriction of T cell Functions

Earlier studies clearly showed that the K, D and I-regions of the murine MHC coded for lymphocyte surface alloantigens (see Table 1.1), but the biological significance of these gene products was obscure. The finding which had provided the clue as to the normal biological function of these alloantigens was the demonstration that the H-2K and H-2D antigens impose constraints on the recognition of foreign antigens (e.g., viruses) by cytotoxic T (Tc) cells (Zinkernagel and Doherty 1974). In essence, Tc cells can only recognize and lyse target cells sharing the H-2K or H-2D antigens (plus foreign antigen) of the stimulator cells-a phenomenon referred to as "H-2-restriction". These findings imply that the H-2K and H-2D antigens play a critical role in the differentiation of self from non-self. Subsequent to the studies with Tc cells it has been shown that delayed-type hypersensitivity and T helper cell responses are also "H-2-restricted", although these responses usually entail the recognition of Ia, rather than H-2K and H-2D molecules and foreign antigens. The role of the H-2 restriction phenomena in T cell cytotoxicity, delayed-type hypersensitivity and T-B collaboration will be briefly outlined below.

2.1 T cell Cytotoxicity

The H-2-restriction phenomenon was originally described

by Zinkernagel and Doherty in their analysis of the destruction of virus infected target cells by Tc cells (Zinkernagel and Doherty 1974). In their system, they demonstrated that CBA/H (H-2^k) mice infected with lymphochoriomeningitis (LCM) virus generated Tc cells which only killed LCM-infected target cells, but not uninfected H-2^k cells, or target cells of non-H-2^k haplotypes infected with LCM. It therefore appeared that H-2 compatibility between Tc and target cells was required for lysis to occur. Further analysis of H-2-restricted Tc cells using inbred, congenic and recombinant mouse strains revealed that Tc cells generated during viral infections only recognized syngeneic K and D gene products in association with viral antigens (Zinkernagel and Doherty 1979). Subsequently, Tc cells specific for minor histocompatibility antigens (Bevan 1975) such as H-Y (Gordon et al. 1975; Schrader et al. 1975), chemically modified self antigens (Shearer et al. 1975), virus transformed tumours (Black et al. 1976) and a wide range of viruses (Zinkernagel and Doherty 1979) have been shown to be H-2 restricted, only lysing target cells bearing the H-2K and H-2D antigens (plus foreign antigen) of the stimulator cells.

Although the I-region was initially thought to play little, if any role in the H-2 restricted Tc responses, other studies have shown that Tc cells can recognize TNP plus self I-region products on target cells. However, these Tc responses are much weaker than that directed against TNP plus H-2K or H-2D antigens (Wagner et al. 1977). Thus, the H-2K H-2D and Ia antigens appear to share some functional properties and, in fact, Ia antigens appear to act as "restriction structures" for delayed-type hypersensitivity and helper T cells (see below).

From extensive studies it is now clear that the

H-2K and H-2D antigens play a key role in the recognition of antigen on cell surfaces by Tc cells. In explaining the significance of the requirement for MHC antigens in the Tc response to virus-infected cells it has been suggested that the presence of MHC antigens in close association with virus specific molecules enables Tc cells to identify and kill virus-infected cells while ignoring an excess (and blocking of virus receptors by) free virus (Howard 1980).

On the other hand, the recognition structures used by Tc cells to discriminate between MHC antigens alone and MHC antigens plus foreign antigen (termed antigen X) have not been characterised, and remain a major area of speculation. The two most widely discussed models are (i) the 'altered self' model where MHC molecules and foreign antigen interact to form a new antigenic determinant recognized by Tc cells and (ii) the 'two receptor' model where Tc cells carry two receptors, one that recognizes self MHC and the other the foreign antigen. There is experimental evidence and theoretical arguement both for and against these two models (reviewed by Zinkernagel and Doherty 1979), and the issue is far from being resolved. The molecular basis of H-2 restriction is further complicated by the fact that any model should explain the high proportion of lymphoid cells that can respond to MHC antigens (Lindahl and Wilson 1977) and the profound effect of MHC structures both inside and outside the thymus on the specificity of MHC-restricted recognition (Zinkernagel and Doherty 1979; Blanden 1980).

2.2 Delayed-type Hypersensitivity

The first evidence for a H-2 restricted DTH response was reported by Miller et al.(1975). In order to achieve successful transfer of DTH, they found that there was always a requirement for

H-2 compatibility between the donors of sensitised T cells and the naive recipients. In the case of soluble protein antigens such as fowl gammaglobulin, keyhole limpet haemocyanin, and synthetic polypeptides such as GAT, this H-2 compatibility was mapped to the I-A subregion of the H-2 complex. However, when contact chemicals such as dinitroflurobenzene (DNFB) were used as the sensitising agent, sharing of the K, D or I region between the donor T cells and the naive recipient was found to be sufficient for DTH transfer (Vadas et al. 1977). In all these systems it was thought that the restriction required H-2 compatibility between the sensitised DTH cells and the antigen presenting cells, probably an Ia bearing macrophages or epidermal cells.

Evidence has accumulated during recent years to suggest that, depending on the antigen used, K, D or I-region molecules can act as the restriction structures for DTH responses. Thus DTH T cells generated to a variety of non-replicating antigens such as proteins and synthetic polypeptides (Miller 1978a), haptens such as azobenzene-arsonate (Bach et al. 1978), and 4-hydroxyl-3 nitrophenyl acety (NP) (Weinberger et al. 1979) and H-Y antigens (Liew and Simpson 1980) were found to be I-region restricted. However, I-region restricted DTH was generated when NP was coupled to a protein carrier, whereas NP-coupled to syngeneic cells preferentially elicited D-region restricted DTH (Sunday and Dorf 1981).

In the case of replicating antigens such as viruses, it appears that the ability to integrate into cell membranes in an appropriate manner may determine whether the DTH response is K, D or I-region restricted. Thus, DTH to LCM was K, D but not I-region restricted (Zinkernagel 1976) whereas Leung and Ada (1980) have demonstrated that DTH to UV-irradiated (non-infectious) influenza

virus was analogous to DTH to a protein antigen in being I-region restricted. On the other hand, Leung and Ada (1980) observed that when infectious influenza virus was used for both sensitization and elicitation of DTH, two populations of DTH cells could be generated and detected: one was K, D-region restricted, the other I-region restricted. These findings were subsequently supported by the findings of Ertl (1981) who showed that fusion-positive (infectious or UV-irradiated) Sendai viruses generated DTH T cells that were either K, D or I-region restricted, whereas fusion-negative Sendai viruses only stimulated I-region restricted DTH T cells. Two additional reports further support the concept that DTH T cells can be K, D or I -region restricted. Thus, Weiner et al. (1980) have shown that cells mediating DTH to infectious reoviruses were K, D or I-A-subregion restricted whereas Nash et al. (1981b) have reported that DTH responses to Herpes Simplex virus were only I-A subregion restricted.

Thus, it appears that the MHC-restriction of DTH responses to viruses very much depends upon the nature of the virus used. Although the significance of these findings is not entirely clear, it appears likely that non-infectious viruses, like UV-irradiated influenza virus and fusion-negative Sendai virus, were processed like soluble protein antigens by macrophages so that an I-region restricted DTH response was elicited whereas infectious viruses produce virion components integrated into cell membranes and consequently generate K and D-region restricted DTH responses. The interesting aspect from these studies is the degree of functional heterogeneity of the different T cell subsets generated in response to viruses. Thus an immediate question raised is whether a K, D-region restricted T cell mediating DTH activity could also have CML

function. The most unequivocal answer to this possibility has been reported by Lin and Askonase (1980) in that a cloned Tc cell line mediating a DTH reaction is also active against influenza virus-infected target cells. In addition, a T cell clone which has lost its DTH function is also found to be unable to act as an effector cell in CML (Lin and Askonase 1981). These findings thus suggest that a single T cell can mediate two functions -- DTH and CML.

2.3 T-B Collaboration

One of the earliest thoroughly studied examples of genetic restrictions was the demonstration that helper T cells (T_H) and B cells must be compatible at the I-A subregion for successful co-operation in antibody responses (Katz and Benacerraf 1972; Katz 1976; Sprent et al. 1980). In this system the restriction appeared to occur at the T_H cell-macrophage level as when syngeneic but not allogeneic, macrophages and T cells were cocultured, T_H cells were activated. The H-2 region involved in this interaction was subsequently shown to be the I-A subregion. Such a macrophage-lymphocyte interaction may be mediated by soluble factors such as genetically restricted factor (GRF), a complex of I-region associated (Ia) antigen and the exogenous antigen (Erb and Feldmann 1975 b, c) or through direct cell contact. In either case T_H cells appear to recognize a complex of self Ia antigens and foreign antigen, analogous to the H-2K and H-2D restricted behaviour of Tc cells.

Although it is generally agreed that T_H cells exhibit H-2 restricted recognition in their inductive phase, whether a similar H-2 restriction pattern exists in the delivery of help from T cells to B cells is still unclear. Many investigators argue that the

delivery of the helper signal from T cells to B cells depend upon cell-to-cell contact, and is H-2 restricted (Sprent 1978; Yamashita and Shevach 1978), whereas others have reported that the delivery of T cell help is not H-2 restricted (McDougal and Cort; Singer et al. 1979) and is mediated by soluble factors.

More recently, I-A subregion restricted T_H cells have been demonstrated to be involved both in the in vivo (Zinkernagel et al. 1978) and in vitro generation of anti-viral Tc cells (Pang et al. 1976; Kreeb and Zinkernagel 1980). In these systems, H-2 restriction appears to occur at the T_H-macrophage interaction rather than in the activation of Tc cell precursors by T_H cells (Ashman and Müllbacher 1979)

3. THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX (HLA)

The human equivalent of the mouse H-2 complex is the HLA system. Like the search for H-2, the search for histocompatibility antigens in man started as an analysis of blood group antigens on leucocytes. Early studies by Dausset (1958) revealed that human peripheral blood leucocytes could be agglutinated by serum of polytransfused individuals, and this led him to define an antigen called MAC. Soon after its discovery, independent findings were made by van Rood and Payne (van Rood, Ernisse and van Leeuwen 1958, Payne and Rolfs 1958) that leucocyte agglutinins were produced by foetal-maternal stimulation. Using such sera together with a statistical approach based on the 2x2 associations of serum reactions on a random panel of cells, van Rood (1962) defined a two allele system which he called group 4. Following further development of these statistical methods by Bodmer, Payne and colleagues (Payne et al. 1964), an independent allelic system of antigens called LA (L for leucocytes

and A for the first locus) was defined. Later, more antigens associated with the LA and 4 groups were defined and it became clear that they fell into two series of alleles at two closely linked loci, the LA and 4 loci respectively (Curtoni, Mattiuz and Tozi 1967; Terasaki 1970). The combined system, by international agreement under a WHO committee, was given the name HLA meaning H for human, L for leucocyte and A for the first system. Subsequently, the system became more complex because many more constituent loci of the HLA were defined: the LA and 4 loci were renamed A and B respectively with the prefix reserved to describe the whole system.

3.1 The Genetic Map of HLA

Based on the fact that the HLA-D locus in HLA corresponds to the I-region of the H-2 complex, which controls the *in vitro* mixed lymphocyte culture (MLC), it is customarily thought that the H-2K locus corresponds to the HLA-B locus, while H-2D corresponds to HLA-A. The mapping of several complement (C') controlling regions in both the H-2 and HLA complexes has assisted in the assignment of these two systems. Thus the S-region of H-2 and C4 of HLA represent corresponding loci, each coding for the fourth component of complement, and should be aligned. The H-2D and the I-region of the H-2 complex would then align with the HLA-B and the HLA-D of the HLA, respectively (Fig. 1.3). This new analogy was extended recently by Bodmer (1980a) to include the parallel between H-2L and HLA-C. It was also suggested that the gap between HLA-A and HLA-C might contain the equivalent of the Qa and T1 sets of loci (Bodemer 1981).

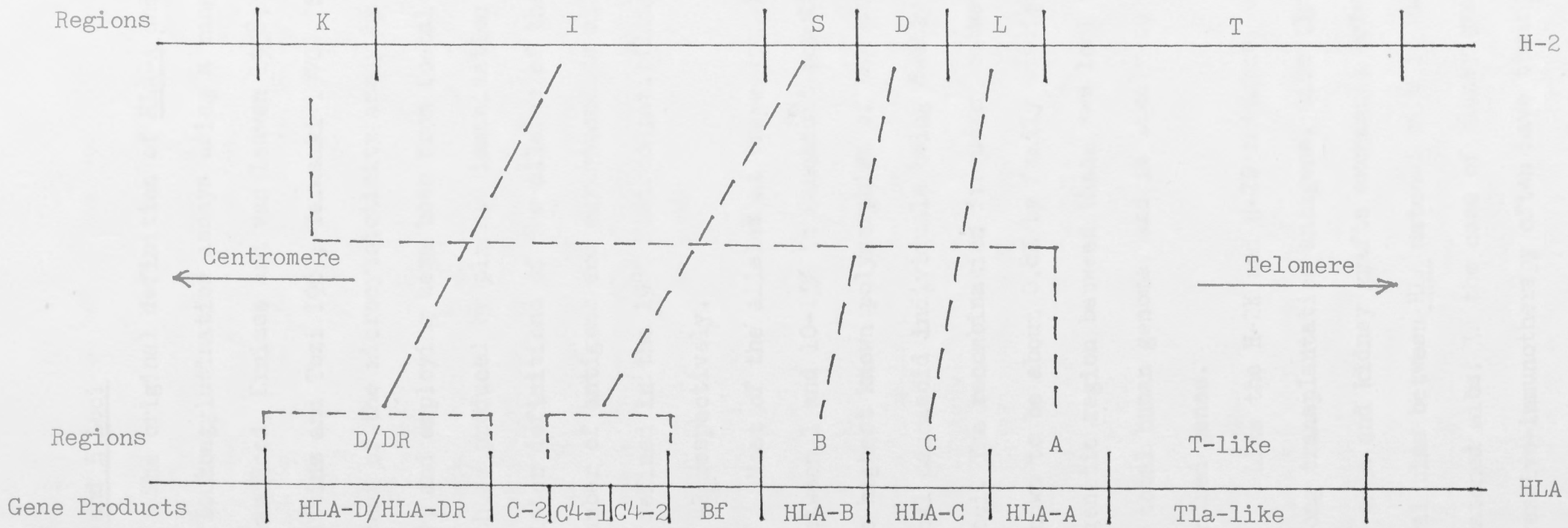

Fig. 1.3 Genetic map of the human HLA as schematically compared to the genetic map of the murine H-2 (modified from Klein 1981; Bodmer 1981).
The genetic products of the HLA are: C2: 2nd component of complement;
C4: 4th component of complement; Bf: properdin factor B; Tla-like: thymus-leukaemia antigen-like

3.1.1 The HLA-A and B Loci

The original definition of HLA-A and B loci came from studies based on leukoagglutination assays using a range of antisera (Dausset, 1958, van Rood, Ernisse and van Leuwen 1958; Payne et al. 1964; Curtoni, Mattinz and Tozi 1967; Terasaki 1970). Subsequently, with the development of the microcytotoxicity assay by Terasaki and McClelland (1964), and employing sera both from foetal-maternal stimulation and those produced by planned immunization of human volunteers, all later definitions of the alleles of these two loci were made. The number of antigens and corresponding alleles of the HLA-A and B loci defined in the 1980 Histocompatibility Testing Workshop were 20 and 42 respectively.

Most of the alleles at these loci have gene frequencies of between 1 and 10-15% in Caucasoid populations. These two loci have the highest known polymorphism of any human gene, more than 75% of typical Caucasoid individuals being doubly heterozygous at the A and B loci. The recombination frequency between the HLA-A and B loci was shown to be about 0.8% by family studies (Bodmer 1978). Thus the genetic region between these two loci is about 1/3,000th of the total human genome, and is likely to include at least several hundred genes.

Like the H-2K and H-2D antigens, the HLA and B antigens are strong transplantation antigens, directly controlling the rejection of skin and kidney grafts exchanged between sibs. Thus the graft survival time between HLA matched sibs is much longer than between unmatched sibs. In the case of kidney grafts, patients kept under suppressive-immunotherapy often have their grafts surviving as long as if the kidney donor was an identical twin (Singal, Mickey and Terasaki 1969). By contrast, matching of the HLA-A and B antigens

between unrelated donors and recipients has had little effect on graft survival, although recent studies suggest HLA-DR matching does significantly improve survival of kidney allografts (Ting and Morris 1980; Svejgaard 1982).

3.1.2 The HLA-D(R) Locus

The HLA-D locus codes for two types of cell surface determinants, namely: D determinants which are involved in inducing T cell proliferative responses, and the D-related, or DR determinants that induce alloantibody production. At present, it is still unclear whether there is more than one locus in the HLA-D region coding for the DR antigens (Dick 1980). The relationship between D and DR antigens is unclear, although most current evidence tends to suggest that they are present on the same gene product (Thorsby 1979). The 1980 International Histocompatibility Workshop formally defined 12 alleles for the HLA-D gene, and 10 alleles for the HLA-DR gene.

Due to experimental limitations, HLA has not been explored in as much detail as the murine MHC. Genetic loci other than HLA-A, B, C and DR which are assigned with certainty in the HLA system are the gene for properdine factor B (B_f), a component of the alternative complement pathway (Allen 1974), and genes controlling the second complement (C2) and fourth (C4) components (Alper 1976; Teisberg et al. 1976).

3.2 Properties of the HLA-A, B, C and DR Antigens

3.2.1 Tissue Distribution of HLA Antigens

HLA-A, B and C antigens are expressed in most tissues except on red blood cells, sperm, trophoblasts and certain

tumour cell lines such as Daudi, Burkitt's lymphoma (Nilsson et al. 1974; Goodfellow et al. 1975), colon carcinoma (Lovo) (Bradsky et al. 1979), and some breast epithelial cell lines (Bodmer 1981). The lack of HLA-A, B and C antigens on sperm presumably protects them from cellular attack in the female after fertilization (Law and Bodmer 1978, Bradsky et al. 1979). The lack of HLA antigens on the trophoblast, on the other hand, may protect the trophoblast from cellular attack so that the foetus can survive as an allograft (Goodfellow et al. 1976; Barnstable and Bodmer 1978; Faulk and McIntyre 1981). Similarly, the lack of expression of HLA-A, B and C antigens on tumour cell lines may enable these tumours to escape from immune attack by effector T lymphocytes (Arce-Gomez et al 1978).

Like the Ia antigens in mice, HLA-DR antigens show a restricted cellular distribution, being present on macrophages, B lymphocytes, epidermal cells and possibly sperm, but usually lacking from T cells, and not being detected on erythrocytes and platelets (Thorsby et al. 1977, 1978; Albrechtsen and Lied 1978; Sandrin et al. 1979). Under certain circumstances, however, HLA-DR determinants are expressed on T lymphocytes, such as Con A T blasts (Albrenchtsen et al. 1977a) and on suppressor T cells generated in mixed lymphocyte cultures (Hirschberg and Thorsby 1977). It should also be noted that, like murine Ia antigens, there is probably a carbohydrate-defined family of HLA-DR antigens that are expressed as glycolipids in serum and on cells (Sandrin et al. 1981).

3.2.2 Methods of Isolation

Initial attempts to purify HLA-A antigens from human splenic lymphocytes were made by Sanderson and Batchelor (1968). Their method, however, has subsequently been shown to be impractical,

only very small amounts of these antigens being obtained. Currently the preferred source of HLA antigens is from lymphoblastoid B cell lines such as BR18 which expresses HLA-A1, A2, B8, B13, CW2 and DRW2 specificities (McCune et al 1975).

Similar to the solubilization of H-2 antigens, the principle methods for extracting HLA antigens are 1) the use of papain to cleave off the membrane proteins (Shimada and Nathenson 1967; Parham et al. 1977; Terhost et al. 1977); or 2) the solubilization of membrane antigens by weakly ionic or non-ionic detergents. The former methodology yields HLA fragments retaining their allo-antigenicity whereas the latter gives intact HLA antigens (Snary et al. 1974; Strominger et al. 1975).

A simple and efficient method for purifying intact HLA-A, B, C and DR antigens had been described by Bridgen et al. (1976). Briefly, the plasma membrane of BR18 cells were solubilized in sodium deoxycholate, and the detergent lysate fractionated by gel filtration. The glycoproteins from the gel filtration column were then selectively absorbed onto Lens culinaris lectin-Sepharose in sodium deoxycholate and eluted off using methyl-α-D-mannopyranoside. Possibly due to the heterogeneity of the HLA-C antigens, half of it was weakly bound to the lectin-Sepharose and did not require sugar elution. Such a scheme gave about a 1300-fold purification in each of their antigenic activities.

The isolation of HLA-DR antigens is essentially identical to that of the murine Ia antigens in that it also involves the immune-precipitation of ^{125}I-labelled detergent-solubilized B cell lymphoblastoid plasma membrane with specific allo-antisera.

3.2.3 Basic Structure of the HLA-A, B, C and DR Antigens

The HLA-A and B antigens are structurally similar to the H-2K and H-2D molecules in that each has a molecular weight of about 44,000 daltons, and is non-covalently associated with β2-microglobulin on the cell surface (Tanigaki and Pressman 1974; Strominger et al. 1976; Barnstable et al. 1978). A hypothetical model of the HLA-A and B antigens with regard to their arrangement in the cell membrane is shown in Fig. 1.4 (a). The 44,000 dalton heavy chain is comprised of three domains, α1, α2 and α3 while β2-microglobulin constitutes the fourth domain. The heavy chain transverses the cell membrane once with about thirty amino acids forming a hydrophilic carboxyl-terminus facing the cytoplasm (Walsh and Crumpton 1977). This C-terminal portion bears one (may be two)-SH group(s) and it has been postulated that the opening and closing of these disulphide bridges under physiological conditions may provide the necessary signal from the outside to the inside of the cell (Strominger et al. 1976). The second domain of the heavy chain possesses a single asparagine-linked oligosaccharide unit of about 3,000 dalton molecular weight about 100 residues from its N-terminus (Parham et al. 1977). Both the first and second domains of the heavy chain probably carry the allotypic determinants (Strominger et al. 1976).

Comparison of the N-terminal amino acid sequence of HLA-A and B antigens revealed that their 3 domains are highly homologous to each other (Bridgen et al. 1976; Terhost et al. 1976), as well as to both the β2-microglobulin and the immunoglobulin (Ig) constant region domains. Sequence variation in the HLA-A and B antigens are found predominantly in their α1 and α2 domains. These

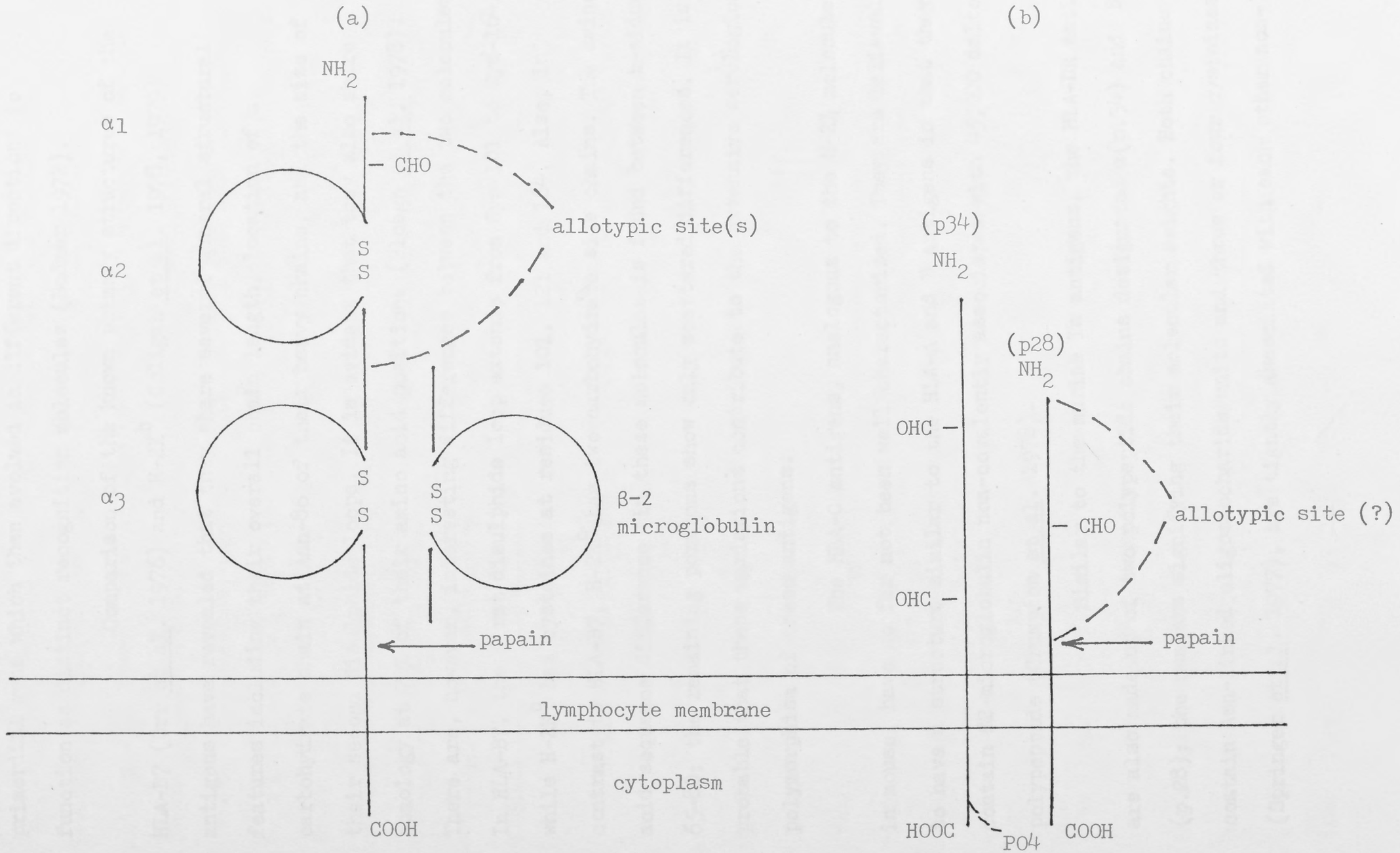

Fig. 1.4 Schematic representation of HLA-A (B) and DR molecules bound to the lymphocyte membrane. The HLA-A or B molecule (a) comprises of four domains: α1, α2 and α3, while the β2-microglobulin comprises the 4th domain. The HLA-DR molecule comprises of the α and β polypeptides which are non-covalently associated with each other (b). S S: Cys-Cys bond; CHO: carbohydrate (adapted from Strominger 1977; Owen and Crumpton 1980)

results suggest that the HLA and the Ig genes have arisen from a common primordial gene which then evolved in different directions to function as distinct recognition molecules (Bodmer 1972).

Comparison of the known primary structures of the HLA-B7 (Orr et al. 1976) and H-2K^b (Coligan et al. 1978, 1979) antigens have revealed that they share several general structural features including their overall chain length, location of a carbohydrate chain at Asn-86 of their heavy chains, and the size of their second disulphide loops. It is evident that they also share homology at 70% of their amino acid positions (Ploegh et al. 1978). There are, however, interesting differences between the two molecules. In HLA-B7, the first disulphide loop extends from Cys-101 to Cys-164, while H-2K^b has cysteines at residues 101, 121 and 164. Also, in contrast to HLA-B7, H-2K^b has two carbohydrate side chains. The amino acid sequence difference in these molecules is found between residues 65-80; the remaining portions show only scattered differences. It is probable that these variations contribute to the extensive serological polymorphism of these antigens.

The HLA-C antigens, analogous to the H-2L molecules in mouse, have so far not been well characterized. They are believed to have a structure similar to the HLA-A and B antigens in that they contain β2-microglobulin non-covalently associated with 45,000 dalton polypeptide (Crumpton et al. 1978).

Similar to the murine Ia antigens, the HLA-DR antigens are also made up of two polypeptide chains designated α(p.34) and β (p.28); the numbers signifying their molecular weights. Both chains contain Asn-linked oligosaccharide units and three or four cysteines (Springer et al. 1977), are tightly associated with each other non-

covalently, span the lipid bilayer of the plasma membrane, and the chain is phosphorylated at the carboxyl-terminus. The molecule, as viewed in the membrane is shown in Fig.1.4 (b). Chemical analysis of DR antigens from several B-cell lines and a chronic lymphocytic leukaemia (Ikeman et al. 1978) revealed that the β chains varied markedly among these cell lines, thus suggesting that they are structurally polymorphic. The α chains, on the other hand, were identical in all the cell lines tested; so have limited, if any polymorphism. At present, there is some evidence suggesting that an invariant chain (Ii) of molecular weight 31,000 daltons (analogous to the Ii associated with the murine I-E/C subregion antigens) may be associated with the DR antigens (Owen and Crumpton 1980).

3.2.4 Biosynthesis of the HLA-A and B Antigens

The biosynthesis of the HLA-A and B antigens has been examined both in vitro and in vivo (Ploegh et al. 1979; Krangel et al. 1979; Owen and Kissonerghis 1980), and it has been shown that their biosynthesis closely resembles that of the H-2K and H-2D antigens. Studies carried out in vivo using the human B lymphoblastoid cell lines T5-1 (Krangel et al.1979) and BR18 (Owen and Kissonerghis 1980) have shown that the heavy chain of the HLA-A and B antigens was first synthesized as a precursor molecule carrying high mannose oligosaccharides in the microsomal membrane. Association of the heavy chain with β2-microglobulin then occurred, and this was followed by the conversion of the high mannose sugar to the complex form involving the stepwise removal of glucose and mannose residues and the addition of the terminal sugars of the complex oligosaccharides (Tabas et al. 1978; Kornfield et al. 1978). The matured HLA-A and B antigens were then intracellularly transported from the endoplasmic reticulum of

the microsome and finally expressed as membrane determinants.

3.2.5 HLA Restriction of Human T Cell Functions

Similar to the H-2 restriction phenomena studied extensively in mice, HLA-restriction has been described for a variety of T cell responses in man. Thus, the proliferative response of BCG sensitised T cells to PPD requires that the soluble antigen be processed and presented by macrophages sharing HLA-DR determinants with the T cells (Bergholtz and Thorsby 1977). In this system, anti-DR antisera were shown to inhibit the PPD induced proliferative response provided they recognized HLA-DR antigens shared by the macrophages and the T cells. No inhibition was observed when the antisera recognized DR antigens carried by the T cells and not the macrophages, while the inhibition was significantly less if the antisera only recognized DR determinants expressed on the macrophages but not on the T cells (Bergholtz and Thorsby 1977; Berholtz and Thorsby 1978). In contrast, HLA-DR restriction between T and B cells, in the absence of macrophages, in inducing PPD T cell proliferation has not been demonstrated (Bergholtz anf Thorsby 1979b).

The first demonstration that human Tc cells exhibit HLA-restriction was reported by Goulemy et al. (1977) for anti-H-Y Tc cells. In a female patient who had received a bone marrow transplant from her HLA-identical brother, her lymphocytes were found to kill male target cells if they shared the HLA-A2 antigen. Subsequently, HLA-A2 has been implicated as the restriction antigen in the killing of DNP-coupled cells by DNP-specific Tc cells (Dickmeiss et al. 1977).

Of the HLA-restricted anti-viral Tc cell responses reported, studies on influenza virus-specific Tc cells have been

the most extensive. It was found that the sharing of either HLA-A or HLA-B locus antigens between the effector and target cells was sufficient for lysis to occur (McMichael and Askonase 1978). This finding was, therefore, consistent with the murine studies where compatibility in the H-2K and/or H-2D region(s) was sufficient for lysis of targets by virus-specific Tc cells (Zinkernagel and Doherty 1980). Unlike the murine influenza virus specific Tc cells, however, HLA-C locus antigens (H-2L like) were found to be not involved (McMichael 1978, 1980; Shaw and Biddison 1979).

So far, HLA-restricted Tc cells specific for other viruses have not been well documented. In fact, T cells obtained from patients with acute infectious mononucleosis were found to lyse Epstein-Barr virus (EBV) infected target cells in a non-HLA-restricted manner (Royston et al. 1975; Svedmyr and Jondal 1979; Tursz et al. 1977). However, if T cells from previously EBV infected individuals were stimulated in vitro with EBV, the Tc cells generated were shown to only lyse HLA-A or B antigen compatible autologous target cells infected with EBV (Moss et al. 1979). Similarly, HLA-A or B antigen compatibility was required for peripheral blood leucocytes (PBL) taken from children with acute measles to lyse measles virus infected PHA stimulated lymphocytes (Kreth et al. 1979). On the other hand, in studies where PBL from normal donors were sensitised with measles virus infected stimulators, the killing of virus infected targets by the effector T cells generated has so far been reported to be non-HLA restricted (Ewan and Lachmann 1977; Wright and Levy 1979).

Collectively these data suggest that, like the mouse, MHC antigens in man also play a key role in the recognition

of foreign antigens. The apparent lack of MHC-restriction in several systems in man probably represents defects in the experimental systems used (e.g., lack of good surface markers for Tc cells, difficulties in controlling genetic differences, etc.) rather than a fundamental difference in antigen recognition by human and murine T cells.

3.2.6 HLA and Disease

With the clear evidence in the mouse that the H-2 complex controls a range of immune response (Ir) genes and can determine susceptibility to viral leukaemogenesis and other diseases (Klein 1978), attempts have been made to correlate certain HLA antigens with disease susceptibility. At present, a number of studies have associated certain HLA-B locus specificities with disease incidence. The best known example of this analysis is the demonstration that HLA-B27 is associated with a rheumatological disorder called ankylosing spondylitis (Brewerton et al 1973; Schlosstein et al. 1973). In addition, B27 has been reported to be associated with Reiter's disease and susceptibility to bacterial infections (Svejgaard and Ryder 1976). Certain HLA-B locus antigens have also been described as being associated with various immune hyperreactivity states. For example, certain HLA-B antigens are linked to Ragweed hay fever (Blumenthal et al. 1974) while HLA-B8 positive individuals have been reported to have a higher incidence of autoimmune diseases (Ryder and Svejgaard 1976; Cudworth and Wooddrow 1976; Rubinstein 1977). There is also some evidence that HLA-B8 positive patients have a better prognosis in Hodgkin's disease (Falk and Osoba 1974), and leukaemic diseases (Rogentine et al. 1973; Oliver et al. 1977). There are some reports of HLA

being linked to malignancy, such as the association between HLA-BW35 and Hodgkin's disease (Amiel 1967), but the only accepted significant association of a HLA antigen with a malignant disease is HLA-BW46 with nasopharngeal carcinoma (Simons et al. 1974) where a herpes-like virus may be implicated.

There are a limited number of reports associating disease susceptibility with the HLA-A and HLA-C loci. For example, HLA-CW6 was reported to be associated with paranoid schizophrenia (Ivanyi et al. 1976), whereas HLA-CW6 was linked with psoriasis, a disease with no known immunological basis (Histocompatibility Testing 1977). The HLA-A locus has been associated with idiopathic haemachromatosis (Histocompatibility Testing 1977) and a deficiency in the second component of complement (C2) has been frequently associated with HLA-A10. Other HLA antigens such as DW2, BW21 and BW40, however, were also linked to this disorder (Cukrova et al. 1977).

Finally, there are two disease susceptibilities that have been linked to the HLA-D locus, namely rheumatoid arthritis with the HLA-DR4 specificity (Stastny 1975) and multiple sclerosis with HLA-DR2 (Bodmer et al. 1977).

One problem with this approach is that many of the associations between HLA and disease have not been highly significant. In fact, the possibility exists that an unrelated gene, which is in linkage disequilibrium with a particular HLA gene, produces disease susceptibility (Cudworth and Wooddrow 1976; Rubinstein 1977).

4. THE AUTOROSETTING PHENOMENON

Before embarking on the experimental chapters of this thesis it is appropriate, at this stage, to review the current knowledge of the phenomenon analysed in the thesis, namely autorosetting.

The binding of autologous erythrocytes by murine lymphoid cells was first described by Micklem and Asfi (1971) in their attempts to detect B cell clones that carried Ig receptors for self antigens. Subsequently, numerous reports appeared demonstrating that murine lymphocytes could rosette with both autologous and allogeneic erythrocytes (Sandilands et al. 1974; Charriere and Bach 1975; Braganza et al. 1975; Kolb 1977). However, different research groups detected markedly different proportions of autorosetting lymphocytes. Such differences certainly reflect the conditions of autorosetting, a point which will be discussed in detail later in this chapter.

In this section, I will review our current knowledge of the autorosetting system. Whether binding of xenogeneic erythrocytes by lymphoid cells represents a similar system of recognition is unknown and, consequently will not be discussed in this review.

4.1 Conditions for Autorosetting

As mentioned above, the conditions of autorosetting are critically important and can markedly affect the proportion of autorosetting cells detected. For example, estimates of the percentage of autorosetting cells in adult mouse thymus have ranged from 0.3% (Micklem and Asfi 1971) to 30% (Kolb 1977).

Three different factors have been shown to have a profound effect on autorosetting. First, the erythrocyte:lymphoid cell ratio is important. Thus using an erythrocyte:lymphoid cell ratio

of 2.5:1, about $400/10^6$ lymph node cells, and $3000/10^6$ thymocytes in normal 6 month-old CBA/H mice autorosetted (Micklem and Asfi 1971). Using a much higher ratio of erythrocyte to lymphocytes (128:1), a higher number of autorosetting cells was detected in murine thymus (2.6%) and spleen (1%) (Charriere and Bach 1975). Subsequently, erythrocyte:thymocyte and erythrocyte:spleen cell ratios of at least 20:1 and 50:1 respectively were found to be required for optimal autorosetting (Steele and Cunningham 1980).

The second factor affecting the number of autorosettes recorded has been the method used to resuspend the lymphoid cell-erythrocyte pellet prior to examination for autorosettes. Low percentages of autorosettes were detected when the cell pellets are resuspended by mechanical rotation at 10-20 rpm on a roller for 2 min at room temperature (Micklem and Asfi 1971; Charriere and Bach 1975; Braganza et al. 1975; Primi et al. 1979). Subsequently, the number of autorosetting cells was found to decrease as the vigour of resuspension increased (Kolb 1978; Steele and Cunningham 1980). For resuspending autorosettes, gentle methods such as using short pasteur pipetttes (Kolb 1978) or gentle tapping (Steele and Cunningham 1980) were recommended.

The third variable that influence aotorosetting is the presence of factor(s) in serum that strongly inhibit autorosette formation (Kolb 1977). Many studies have not taken this variable into account.

On the other hand, autorosetting was shown to be independent of metabolic activity and cytoskeleton function as it was not influenced by metabolic inhibitors such as sodium azide, and sodium cyanide or by the cytoskeleton inhibitors colchicine and vinblastine (Kolb 1978). Furthermore, dead cells autorosetted (Kolb

1977). Autorosetting was also found to be independent of the divalent cations Ca^{++} and Mg^{++}, could be formed within the pH range of 5.5-7.5 (Kolb 1978) and could occur at 4-37°C. (Kolb 1978). However, the electrostatic force between lymphocytes and erythrocytes appears to influence autorosette formation as the inclusion of the polyanion heparin (20 units/ml) in serum free medium completely abolished autorosetting, presumably by increasing the electrostatic repulsion between cells (Kolb 1978).

4.2 Nature of Autorosetting Cells

There have been contradictory reports on the nature of the autorosetting cells. Originally, Micklem and Asfi (1971) suggested that autorosetting cells were immature T cells, although this suggestion was based simply on the observation that thymus had a higher incidence of autorosetting cells than lymph node. In subsequent studies Charriere and Bach (1974, 1975) obtained additional evidence supporting the claim that autorosetting cells were immature T cells. They found that autorosetting cells were blocked by anti-Thy-1.2 sera, adhered to nylon wool columns, had a low density, and were sensitive to steroids, all characteristics of immature T cells. Furthermore, higher levels of autorosetting cells were reported in thymectomised mice which could be corrected to normal values by administration of thymic hormones, treatments that presumably influenced T cell differentiation. In addition, recent morphological studies have shown rosetting cells to be large with clear and well developed euchromatin, abundant cytoplasm with many free ribosomes and mitochondria which were characteristics of immature T rather than mature T cells (Charriere and Bach 1979). These autorosetting T cells also have been shown to proliferate when cultured with

Con A (Fournier and Charriere 1978).

A major criticism of the studies described above is that autorosetting conditions were employed that were far from optimal, usually 2% of spleen, thymus and lymph node being shown to autorosette. Using more superior autorosetting conditions Kolb (1977) demonstrated that murine thymus and spleen contained a similar proportion of autorosetting cells, i.e., approximately 30% of the cells autorosetted. In addition, cells from the spleen of athymic nude mice autorosetted, thus suggesting for the first time that B lymphocytes could also autorosette. Subsequent immunofluorescence studies by Primi et al. (1979) demonstrated that 70-80% of autorosetting cells in mouse spleen were Ig^+.

The simplest explanation of these contradictory findings is that immature T lymphocytes autorosette more avidly than mature T lymphocytes and B lymphocytes, hence their preferential detection when autorosetting conditions are suboptimal. The experimental data presented in this thesis is completely consistent with this explanation.

4.3 Ontogeny of Autorosetting Cells

Murine autorosetting cells have been detected in the yolk sac as early as 10-11 days of gestation, and their number could be reduced by exogenous thymic factor (Charriere and Pyke 1980). As the embryo developed, autorosetting cells were detected at 14-days of gestation in the foetal liver, then in the same numbers in the 15-16 day old foetal thymus, and in the 17-18 day old foetal spleen (Steele and Cunningham 1980). Foetal thymus autorosettes reached adult levels at 17-18 days of gestation, and spleen autorosettes at 2 weeks

after birth (Steele and Cunningham 1980).

4.4 Autorosetting in Different Species

There are a limited numbers of studies demonstrating autorosetting lymphoid cells in species other than the mouse. For example, it has been shown that rabbit spleen has the same number of autorosetting cells as the mouse spleen (Kolb 1977). Surprisingly, no autorosetting cells were found in the spleen of rats (Kolb 1977). On the other hand, human thymus contains from 40-75% of autorosetting cells (Baxley et al. 1973).

A thorough study into the autorosetting ability of the peripheral blood leucocytes (PBL) of a variety of species revealed that the cells from hamster, guinea pig, rabbit, rat, mouse and dog lacked autorosetting ability. However, about 7% and 20% of autorosetting cells were found in human and sheep PBL respectively (Braganza et al. 1975). This study was marred, however, by suboptimal autorosetting conditions being used.

4.5 Serum Inhibition of Autorosetting

Several studies have demonstrated that autorosette formation can be blocked by serum (Micklem and Asfi 1971; Charriere and Bach 1974, 1975). By employing serum free Hank's balanced salt solution (HBSS) for autorosetting, Kolb (1977) was able to detect at least twice the number of autorosetting cells in murine thymus and spleen compared to that obtained by other workers. Similarly, the number of autorosetting cells detected in human PBL could be increased 4-9 fold when FCS was replaced by BSA in the rosetting medium (Braganza et al. 1975). Serum inhibitory material was preliminary characterised by Kolb (1977) in that it was non-dialysable, was not

precipitated by 18% sodium sulphate suggesting that it was not an immunoglobulin, and was found to fractionate with serum α-globulins. Recently, the inhibitory material in mouse serum (termed "autorosette inhibition factor" or AIF) was demonstrated to be associated with serum high density lipoproteins (Hsu et al. 1980).

4.6 Nature of the Autorosetting Receptor and Acceptor

Little is known about the membrane structures mediating autorosetting. There is some controversy, however, as to whether the autorosetting receptors on murine lymphoid cells are associated with immunoglobulin (Ig). Among the evidence in support of the receptor being Ig or Ig-associated is the report that lymph node cell autorosettes can be inhibited by pretreating the lymph node cells with anti-Ig sera (Micklem and Asfi 1971). In addition, the autorosetting ability of adult and new-born spleen cells was abolished by anti-Ig capping using a sheep anti-mouse Ig serum (Steele and Cunningham 1980). Autorosettes in thymus, however, were not affected by anti-Ig capping treatment (Steele and Cunningham 1980). In direct contradiction to these findings, anti-Ig capped spleen cells were shown to autorosette, even though the majority of autorosetting spleen cells were demonstrated to be Ig^+ by immunofluorescence (Primi et al. 1979). It seems likely, however, that autorosetting receptors are not Ig-associated and the earlier contradictory results are due to the use of anti-Ig reagents containing autorosette inhibitory factor. When immunoabsorbent purified anti-Ig was used (Primi et al. 1979, chapter 3) the autorosetting receptors were clearly shown not to be Ig.

There has been only one preliminary attempt to

characterize the membrane structures involved in autorosetting, namely the effect of different enzymic treatments on the autorosetting behaviour of thymocytes and erythrocytes (Kolb 1978). The autorosetting ability of thymocytes or spleen cells was abolished by trypsin, while erythrocytes were found to lose their autorosetting ability when treated with trypsin, papain or bromelin. Treatment of erythrocytes or lymphocytes with neuraminidase was found to increase the proportion of lymphocytes autorosetting. This effect was probably due to the reduction of electrostatic repulsion between cells when sialic acid residues where removed (Kolb 1978). From these studies it was concluded that autorosetting represented a protein-protein interaction between lymphocytes and erythrocytes.

4.7 Role of Major Histocompatibility Complex (MHC) in Autorosetting

The role of the murine H-2 complex in autorosetting has been recently investigated by two research groups. By rosetting BDF1 spleen cells, after 24 hrs culture, with erythrocytes from different congenic and recombinant strains of mice, Primi et al. (1979) found that lymphocytes and erythrocytes differing in the H-2 complex failed to autorosette. By examining intra-H-2 recombinant strains of mice, it was found that compatibility at the D region was required for autorosetting. There was also a suggestion from the behaviour of some recombinants that autorosetting was controlled by a separate locus (H-2R) mapping just to the left of the D region.

Charriere et al. (1980) employed two approaches to determine the role of the H-2 complex in autorosetting. First, like Primi et al. (1979), they observed that lymphocytes preferentially bound H-2 compatible erythrocytes. The second approach was to measure the ability of H-2 compatible or incompatible erythrocyte

ghosts to inhibit autorosetting, a study which revealed that H-2 compatible ghosts were the most efficient inhibitors. Furthermore, based on their results from one H-2 recombinant mouse strain they concluded, unlike Primi et al. (1979), that both K and D regions of the H-2 complex were involved in autorosetting.

It should be noted that studies reported in this thesis, that were carried out at the same time as those described above, demonstrated H-2 involvement in autorosetting and directly mapped the gene(s) involved to the H-2L region (see chapter 3).

Thus, three independent studies have clearly demonstrated that the autorosetting receptors on lymphocytes preferentially recognize H-2 compatible structures on erythrocytes. There is some disagreement, however, as to which regions of the H-2 complex are involved, a point which will be discussed in more detail in chapter 3 and 7 of this thesis.

4.8 Functional Significance of Autorosetting Receptors

The functional significance of the autorosetting receptors on lymphoid cells is unknown. There are only two reports that have attempted to correlate autorosetting cells with immune function. The first was by Carnaud et al. (1977), who observed that spleen cells from adult thymectomised mice induced an enlargement of the draining popliteal lymph node following inoculation into the hind footpad of syngeneic recipients (termed "syngeneic graft versus host reaction"). However, when the cell inoculum was depleted of autorosetting cells on a Ficoll/Hypaque gradient there was a significant reduction in the syngeneic G V H reaction, a result suggesting that autorosetting lymphocytes could mediate this response.

In the second study by Tomari et al. (1980), it was shown that those T lymphocytes that responded in an autologous mixed lymphocyte reaction (MLR) were present in the autorosetting subpopulation of T cells. Furthermore, when the T cells that responded in a primary autologous MLR were restimulated it was found that compatibility of the whole HLA complex was required to generate the secondary response. These findings, however, do not prove that the autorosetting receptors actually initiate syngeneic G V H or autologous MLR. Furthermore, the functional significance of the syngeneic G V H reaction and autologous MLR is unknown.

At a more speculative level it has been proposed that the autorosetting cells may mediate autoimmunity in thymus deficient states (Charriere and Bach 1974). The supportive evidences for this proposal include: (i) the observation that the percentage of autorosetting cells in the spleen was increased from 6 days and attained a 20-30 fold increase 3 months following adult thymectomy and (ii) increased levels of autorosetting cells were found in adult 6 week-old nude mice, 4-month old NZB mice and 15 month-old mice which showed thymic atrophy as well as spontaneous auto-antibody formation. Furthermore, these abnormally high autorosetting levels could be returned to normal by the injection of purified thymic hormone bound to carboxy-methyl cellulose to increase its half life. However, as mentioned earlier, in their studies Charriere and Bach only detected a small fraction (10%) of the autorosetting cells due to suboptimal autorosetting conditions. This makes their findings difficult to interpret.

Chapter 2

Materials and Methods

Table of Contents

2.1 Animals

Table 2.1 lists the mouse strains used in this study together with the haplotype origin of their H-2 regions. The mice and Lewis rats were bred at the John Curtin School of Medical Research, the majority under specific pathogen free conditions. F1 hybrid mice were obtained from six different matings over a 4 month period. Mice were used as donors of erythrocytes and/or thymocytes from 8-20 weeks of age.

Congenitally athymic BALB/c nude (nu/nu) mice were obtained from the John Curtin School of Medical Research. Nude mice used for experiments were always supplied with antibiotics (Kanamycin sulfate and tetracycline hydrochloride bought from Bristol-Myers Co., N.S.W) in their drinking water. The beige mutant mice C57BL/6J bg^J/bg^J (bg/bg) and their heterozygous (bg/+) littermates were a gift from Dr. G. R. Shellam, University of Western Australia.

Chimeras were produced as described in detail elsewhere (Sprent et al 1975). The mice were irradiated with a supra-lethal dose of 900-1000 Rads from a cobalt source, and on the same day or 24 hours later were reconstituted with 1.5 - 2.5 x 10^7 foetal (14-16 days) liver cells injected intravenously (i.v.). All chimeras were analysed individually 6-12 weeks later and were shown via complement mediated lysis with anti-H-2 sera, to be 90% repopulated with donor lymphocytes.

2.2 Sera and Antisera

Blood of mice, rats, Guinea Pigs and African Clawed Toad was collected by cardiac puncture. Blood from geese and chickens were obtained by bleeding their wing veins. Peripheral blood of humans and sheep was collected by venous puncture. Blood samples were allowed to clot at room temperature for 30 min at 4°C. Sera were stored at -20°C until use. The serum of Little Brown Bat was kindly donated by Mr. C. R. Tideman, Zoology Department, Australian National University.

Table 2.1

Haplotype Origin of H-2 Regions Carried by Mouse Strains Used in this Study

Strain	Haplotype	Region [a]							
		K	I					S	L/D
			A	B	J	E	C		
C57BL/6, C57BL/10	b	b	b	b	b	b	b	b	b
BALB/c, DBA/2	d	d	d	d	d	d	d	d	d
B10.D2 (M504)	dml	d	d	d	d	d	d	d	d [b]
B10.A (2R)	h2	k	k	k	k	k	d	d	b
B10.A (4R)	h4	k	k	b	b	b	b	b	b
B10.A (3R)	i3	b	b	b	b	k	d	d	d
CBA/H, B10.BR	k	k	k	k	k	k	k	k	k
DBA/1, B10.G	q	q	q	q	q	q	q	q	q
SJL/J, A.SW	s	s	s	s	s	s	s	s	s
A.TH	t2	s	s	s	s	s	s	s	d
B10.AQR	y1	q	k	k	k	k	d	d	d
B10.T(6R)	y2	q	q	q	q	q	q	q	q

a) Haplotype origin of regions according to Klein et al. (1978)

b) The dml mutant carries modified H-2L and H-2D (Demant and Neauport-Sautès 1978).

Anti-H-2L^{d} serum which was raised in (BALB/c-H-2^{dm2} x A.SW)F1 mice against BALB/c lymphoid cells (AS 207) was a gift from Dr. I. F. C. McKenzie. Monoclonal anti-H-2.25 antibody (H100-27.R9) was provided by G. Hammerling. By microcytotoxicity the anti-H-2L^{d} serum had a titire of between 1/16-1/32 on BALB/c spleen cells whereas the anti-H-2.25 antibody gave a titre of 1/20,000 on CBA/H spleen cells.

Monoclonal anti-Thy 1.2 IgM antibody (F7D5) was kindly supplied by Dr. P. Lake. The antibody had a microcytotoxicity titre on CBA/H spleen cells of 1/5000.

2.3 Sugars

The sugars used in the inhibition studies and the suppliers are listed in Table 2.2. These sugars were usually dissolved in phosphate-buffered saline (PBS) at a concentration of 20 mg/ml. The pH of N-acetylneuraminic acid, D-glucosamine, D-glucuronic acid, D-galactosamine, D-mannosamine and D-galacturonic acid was adjusted to 7.2 with 0.1N NaOH before use.

2.4 Enzymes and Periodate

Papain, α-galactosidase (Green Coffee Bean), β-galactosidase (E. coli, grade 111), and α-mannosidase (Jack Bean, Type 111) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Neuraminidase (Vibro cholera, B grade), pronase and Bromelin were supplied by Calbiochem, San Diego, U.S.A. Trypsin-TPCK was purchased from Worthington Biochem. Corp., New Jersey, U.S.A. Sodium metaperiodate was supplied by BDH, Poole, England.

2.5 Anti-macrophage Agents

Carrageenan Type 1 from Irish Moss was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Silica particles of average size distribution 5μ were obtained from Whittaker, Clark & Daniels Inc., New Jersey. Carrageenan was dissolved in physiological saline (10 mg/ml) before 0.2 ml (i.e. 2 mg) being injected intravenously (i.v.) into each mouse. To make up varying dilutions

Table 2.2

Sugars Used for Inhibition Studies

Monosaccharides:

D-glucose [a]
N-acetyl-D-glucosamine
D-glucosamine [b]
D-glucuronic acid [b]
D-galactose [b]
N-acetyl-D-galactosamine
D-galactosamine
D-galacturonic acid [b]
D-mannose [c]
L-mannose
N-acetyl-D-mannosamine
D-mannosamine
D-mannitol
L-fucose [b]
D-fucose
L-rhamnose [c]
D-ribose
N-acetylneuraminic acid

Glycosides:

methyl-α-D-glucopyranoside [b]
methyl-α-D-galactopyranoside [b]
methyl-β-D-galactopyranoside
methyl-α-D-mannopyranoside

Oligosaccharides:

melibiose [b]
raffinose [b]
stachyose
lactose [d]
maltose [b]
mannan
cellobiose [b]
sucrose [d]

a) Ajax Chemicals Ltd., Sydney, Australia

b) Calbiochem, San Diego, California, U.S.A.

c) Fluka AG, Buchs, Switzerland

d) BDH, London, England

Remaining sugars suppied by Sigma Chemical Co., St. Louis, Mo., U.S.A.

of carrageenan, the 10mg/ml stock solution was serially diluted in hot physiological saline. Silica was suspended in physiological saline (10 mg/ml), sonicated for 30 sec using a Branson (Danburg, Conn.) probe-type sonicator (Sonifier B12 model) and immediately 0.2 ml (i.e. 2 mg) injected into each mouse.

2.6 Cell Populations

2.6.1 Preparation of Cell Suspensions

Thymocyte, lymph node and spleen cell suspensions were prepared by gently pressing thymus, lymph node or spleen fragments through wire sieves into cold Hank's balanced salt solution (HBSS) containing 0.5% (v/v) foetal calf serum (FCS). Peritoneal cells were harvested by lavage of the mouse peritoneal cavity with 5 ml of F15 medium lacking FCS. For the collection of bone marrow cells, both ends of the femur bones were cut, and the marrows 'blown-out' with F15 medium lacking FCS using a hypodermic syringes with a 18g needle. All cell suspensions were gently pelleted, cell clumps removed by low speed centrifugation (100g, 30 sec), and the dispersed cells washed twice with cold medium. The thymocytes and spleen cells were then counted and cell viability assessed by trypan blue exclusion. Cells were then diluted to a concentration of 2×10^6/ml in medium.

Blood was collected in Alsever's solution. Erythrocytes were washed three times with saline for 3 min at 2000 rpm and 22°C and diluted to 0.5% or 1% in HBSS containing 0.5% FCS.

2.6.2 Separation of Lymphocyte Populations

Cells bearing surface Ig were removed from lymphoid cell suspensions by forming Ig rosettes and separating the rosetting and non-rosetting cells by centrifugation on Isopaque/Ficoll (Parish et al 1974). Briefly, sheep red cells (SRBC) were coated, via $CrCl_3$, with sheep anti-IG specific for mouse Ig and were mixed with lymphoid cell suspensions. The mixture of SRBC and spleen cells was pelleted by centrifugation, the pellet resuspended

in its supernatant and the rosette-forming cells then sedimented on Isopaque/Ficoll-medium interface. To separate Ig^-, $Thy\text{-}1.2^+$ spleen cells, the splenocytes were first incubated with monoclonal anti-Thy-1.2 antibody (30 µl/10^8 lymphocytes). Cells carrying the Thy-1.2 antigen thus became coated with mouse antibody and therefore both $Thy\text{-}1.2^+$ and Ig^+ cells now rosetted for surface Ig. Using this procedure 92% of B10.BR spleen cells rosetted and the rosettes were depleted in the usual manner on Isopaque/Ficoll (Parish et al 1974). In some cases, spleen cells were depleted of $Thy\text{-}1^+$ cells by being treated with monoclonal anti-Thy-1.2 antibody and guinea pig complement (McKenzie and Parish 1976) and the resulting dead cells removed by Isopaque/Ficoll centrifugation (Davidson and Parish 1975).

2.6.3 Blocking and Capping Surface Immunoglobulin (Ig) on Spleen Cells

Spleen cells were made to a concentration of 10^7 cells/ml in ice-cold balanced salt solution containing 0.5% FCS. Immunoabsorbent purified sheep $(Fab')_2$ specific for mouse Ig was added to a concentration of 50 µg/ml and the mixture incubated for 75 min at either 4°C for blocking, or 37°C for capping. Previous studies have demonstrated this procedure to completely block and clear surface Ig from mouse spleen cells (Parish and McKenzie 1980). Following incubation the cell suspensions were washed twice with ice-cold medium and used for autorosetting (see section 2.10.1).

2.7 Preparation of Subcellular Fractions of Cells

2.7.1 Preparation of Erythrocyte Sonicates

Blood was collected from mice or Lewis Rat in Alsever's solution and washed four times in normal saline. Packed or 50% suspension of erythrocytes in medium were then disrupted by sonication for 10 sec at 30 watts using a Branson (Danburg, Conn.) probe-type sonicator (Sonifier B 12 model). The red cell suspensions were completely transparent after

this treatment. Sonicates were usually prepared on the day of use or stored for no longer than 24 hr. at 4°C.

2.7.2 Preparation of Detergent Lysates of Lymphocytes and Erythrocytes

Spleen and bone marrow cell suspensions were first sedimented on Isopaque-Ficoll to remove erythrocytes (Davidson and Parish 1975). Thymocyte and lymph node cell suspensions were not treated in this manner if they were freed of red cells. The lymphoid cells were then washed twice in cold PBS and used for lysate preparations.

To 1 x 10^8 lymphoid cells suspended in 0.5 ml of cold PBS containing 10^{-3} M phenylmethylsulphonylfluoride (PMSF) was added 0.5 ml of a 1% (v/v) Nonidet P-40 (NP-40) solution. The mixture was incubated for 30 min on ice with occasional shaking. The cell nuclei were then pelleted at 4000 g for 5 min at 4°C and the supernatant spun at 28,000 g for 15 min at 4°C to remove any remaining membrane fragments. The lysate was depleted of NP-40 by adding an equal volume of packed XAD-8 resin beads (Rohm and Hass Co., Philadelphia, Pa) and agitating the lysate-resin mixture for 2 hr at 4°C (Parish et al 1980). The detergent-depleted lysate was then used for haemagglutinating assays.

In the case of erythrocyte lysates, blood was collected from mice and other animals in Alsever's solution and the red cells washed four times in cold PBS before use. 200 µl of packed erythrocytes were lysed in 10 ml of cold distilled water for 10 min on ice. The erythrocyte ghosts were pelleted by spinning the mixture at 28,000 g for 30 min at 4°C. The pellet was then resuspended in 500 µl of

cold PBS containing 10^{-3} M PMSF, 500 µl of 1% NP-40 added and lysis allowed for 30 min on ice. The lysate was then spun at 28,000 g for 30 min at 4°C, the supernatant treated with XAD-8 and used for the inhibition of haemagglutination.

2.7.3 Preparation of Cytoplasmic and Microsomal Fractions of Thymocytes

Thymocytes were suspended at a concentration of 1×10^8 cells/ml in PBS containing 10^{-3} M PMSF and subjected to sonication, in an ice bath, at 30 watts for 5 min using a Branson (Danbury, Conn.) probe-type sonicator. Cell debris was pelleted by centrifugation at 4000 g for 60 min to give a microsomal pellet and freely soluble cytoplasmic components. The microsomal pellet was then solubilised in 0.5% NP-40 as described above (10^8 cell equivalents/ ml).

2.8 Enzymic and Chemical Treatments of Cells

2.8.1 Enzymic Treatment of Thymocytes and Erythrocytes

In all treatments thymocytes were used at 8×10^6 cells/ml and erythrocytes as a 1% (v/v) solution. Thymocytes or erythrocytes in HBSS were incubated separately with 0.2 mg/ml of pronase (pH 7.5), 1 mg/ml of trypsin (pH 8.0) or 1mg/ml of bromelin (pH 7.5). The HBSS was adjusted to the appropriate pH with 1N NaOH prior to use. Cells were incubated with trypsin or bromelin for 30 min at 37°C whereas, in order to minimize cell lysis, pronase digestion was limited to 10 min at 37°C. For papain treatment, cells were incubated at 37°C for 30 min with 0.4 mg/ml of enzyme in PBS containing 1.2% (w/v) L-cysteine and 0.186% (w/v) EDTA.

Thymocytes and erythrocytes were incubated for 30 min at 37°C with the following concentrations of glycosidases: β-galactosidase-

20 units/ml, α-mannosidase-2 units/ml, neuraminidase-10 units/ml, α-galactosidase-0.5 units/ml and 0.6 mg/ml of mixed glycosidases supplemented with 10 units/ml of neuraminidase. All glycosidase treatments were performed in HBSS, except for the mixed glycosidases incubation where a 1:1 (v/v) mixture of PBS and calcium-magnesium saline was used.

With all treatments a control was included where cells were incubated under identical conditions but in the absence of the enzyme or chemical. Controls for the α-galactosidase, β-galactosidase and α-mannosidase treatments also included the equivalent amount of ammonium sulphate added with the enzyme.

2.8.2 Periodate Treatment of Lymphocytes and Erythrocytes

For periodate treatment, 1 x 10^8 lymphoid cells in 1.0 ml of cold PBS were mixed with 4.0 ml of cold PBS containing 21.4 mg of sodium metaperiodate (BDH, Poole, England). The mixture was left on ice for 30 min after which it was washed 3 times with cold PBS. Cell lysates were then prepared as described above.

2.8.3 Glutaraldehyde Fixation of Mouse Erythrocytes

A stock solution of glutaraldehyde (70%) was diluted 1:1 with distilled water, and then to a final concentration of 0.05% (v/v) with PBS. Equal volumes of 0.05% glutaraldehyde solution was then mixed with a 5% (v/v) erythrocyte suspension in PBS, and the mixture left for 10 min at room temperature. The erythrocytes were washed four times with PBS and then twice with PBS containing 0.1% BSA prior to being used for the absorption of thymocyte lysates.

2.9 Lactoperoxidase-Catalysed Iodination of Cells

The method was essentially similar to that described by Cone and Marchalonis (1974). A suspension of thymocytes was prepared, the cells were washed twice in serum-free PBS and then resuspended to a concentration of 2×10^8 cells/ml. For iodination, to 5×10^7 cells in 250 µl of PBS containing 80 µg of lactoperoxidase, was added carrier-free ^{125}I (500 µCi), immediately followed by 30 µl of 12 µg/ml H_2O_2 in PBS. The mixture was incubated on ice for 1 min, another 30 µl of 12 µg/ml H_2O_2 in PBS added, and the mixture further incubated on ice for 1 min. The reaction was then stopped by the addition of 20 volumes of ice-cold PBS. The cells were sedimented by centrifugation at 4°C, washed twice with ice-cold PBS, and finally resuspended in 250 µl of cold PBS. The preparation of lysate from these ^{125}I-labelled thymocytes was identical to that described previously for non-labelled thymocytes.

For the iodination of erythrocytes 20 µl of packed erythrocytes previously washed four times in serum-free PBS were resuspended in 180 µl of cold PBS containing 160 µg of lactoperoxidase, and iodination then carried out as described for thymocytes. Lysis of ^{125}I-labelled erythrocytes was the same as for unlabelled erythrocytes except that preparation of erythrocyte ghosts was omitted.

2.10 Rosetting Assays

2.10.1 Standard Rosetting Assay and Important Assay Variables

The standard autologous, allogeneic and xenogeneic rosetting assay was carried out in the following manner in 96 well U-bottomed microlitre plates (Linbro Chemical Co., New Haven, Conn.).

To 25 µl of each murine lymphoid cell suspension (2×10^6/ml) was added 25 µl of different concentrations of appropriate red cell. The red cell-lymphoid cell mixture was then pelleted by centrifugation at 200 g for 1 min at 4°C. The cell pellets were incubated on ice for 30-60 min before being very gently resuspended with a short pasteur pipette. Methyl violet staining solution (100 µl) (Parish and McKenzie 1978) was then added to the wells, each sample transferred to a haemocytometer chamber and the percentage of rosette forming cells assessed. Usually a minimum of 100-200 thymocytes were scanned for rosettes and when the rosette frequency was < 10% up to 500 thymocytes were examined. Any thymocyte which bound four or more erythrocytes was classified as a rosette.

At this point, several important assay variables should be discussed. First, the erythrocyte concentration (erythrocyte: lymphocyte ratio) markedly affected autorosetting efficiency. It was found that the highest percentage of thymocyte autorosettes was obtained when an erythrocyte concentration equal to or greater than 0.5% was used, whereas a red cell concentration greater than 0.5-1% was required for optimal spleen autorosetting (Table 2.3)

The second variable which influenced autorosetting efficiency was temperature and pH. By performing autorosetting at room temperature (using 0.5% erythrocytes), it was found that the percentage of BALB/c ($\underline{H\text{-}2}^d$) thymocyte autorosettes detected was usually lower than if the assay was carried out at 4°C (25% vs 44%). On the other hand, medium in the pH range 6.8-7.2 was found to be required for the formation of the optimal number of autorosettes (Table 2.4).

Table 2.3

Effect of Erythrocyte Concentration on the Detection of BALB/c ($H\text{-}2^d$) Autorosettes

Erythrocyte Conc. (%)	Erythrocyte Lymphocyte Ratio	% Autorosettes Thymus	% Autorosettes Spleen
		a)	
0.1	5:1	2 ± 0.7	2 ± 0.5
0.2	10:1	8 ± 1.9	2 ± 0.6
0.3	15:1	24 ± 3.1	4 ± 1.5
0.4	20:1	37 ± 3.5	8 ± 1.7
0.5	25:1	44 ± 2.7	12 ± 2.2
1.0	50:1	45 ± 3.4	14 ± 2.4
2.0	100:1	44 ± 2.9	14 ± 3.1

a) Results expressed as % rosetting lymphoid cells ± standard error of mean of three determinations.

Table 2.4

Effect of pH of HBSS on the Autorosetting of BALB/c Thymocytes

pH of HBSS b)	% Thymocyte Autorosettes
	a)
6.0	7 ± 2.1
6.4	25 ± 2.8
6.8	43 ± 3.1
7.0	42 ± 4.0
7.2	44 ± 2.9
7.5	34 ± 3.6
8.0	12 ± 3.4

a) Footnote as in Table 2.3

b) pH of HBSS was adjusted by adding either 1N NaOH or 1N HCl.

The third factor affecting autorosetting efficiency was the incubation time on ice before autorosettes were resuspended (described below) for examination. By performing BALB/c (H-2^d) thymocyte autorosetting using an erythrocyte:lymphocyte ratio of 25:1, it was found that an incubation time of at least 25 min was required for detecting the optimal number of autorosettes (Table 2.5).

Table 2.5

Effect of Incubation Time on the Detection of BALB/c Thymocyte Autorosettes

Incubation Time (min)	% Autorosettes
1	0
5	1 ± 0.5 a)
10	7 ± 1.2
15	19 ± 3.1
20	37 ± 3.4
25	44 ± 3.2
30	43 ± 3.0
60	44 ± 2.8

a) Footnote as in Table 2.3

The fourth factor which was critical in determining the percentage of autorosettes detected was found to be the method employed to resuspend the rosettes. When resuspension was by gentle vortexing for 10 sec at room temperature, low percentages

of autorosettes was always obtained, i.e., 7% vs 43% for BALB/c ($\underline{H\text{-}2}^{d}$) thymocyte autorosetting using an erythrocyte/lymphocyte ratio of 25:1. Subsequently, autorosettes were resuspended by using a short pasteur pipette. By gently pipetting the mixture up and down 8 times, the erythrocyte/lymphocyte mixture was usually homogeneously dispersed, and reproducible numbers of autorosettes were detected, these numbers being higher than reported by other groups who resuspended rosettes with a roller.

As autorosetting was always reported in the literature to have been carried out using HBSS or PBS as medium, it was, therefore, important to determine whether medium had an effect on autorosetting efficiency. By using BALB/c ($\underline{H\text{-}2}^{d}$) thymocyte autorosetting (erythrocyte/lymphocyte ratio of 25:1), it was found that optimal percentages of autorosettes were obtained in HBSS, PBS or physiological saline (0.9%) containing 0.5% FCS (Table 2.6). In contrast, low percentages of autorosettes were detected when Eagle's Minimal Essential Medium (F15) or RPMI-1640 were used. The reason for autorosetting being suboptimal in F15 or RPMI-1640 is unknown, although these media represent complex mixtures of amino-acids, vitamins and cofactors, some of which presumably inhibit autorosetting.

Table 2.6

Effect of Medium on the autorosetting of BALB/c ($\underline{H\text{-}2}^{d}$) Thymocytes

Medium	% Thymocyte Autorosettes
HBSS	44 ± 2.9 a)
PBS	43 ± 3.4
Physiological Saline	42 ± 2.7
F15	6 ± 1.6
RPMI-1640	7 ± 1.4

a) Footnote as in Table 2.3

Finally, it should be noted that normal animal sera contain a potent inhibitor of autorosetting, a point which will be discussed in detail in chapter 6. It was, important, therefore, to perform autorosetting either in serum free medium or in the presence of proteins that did not inhibit autorosetting. It was found that most batches of FCS were not inhibitory, particularly at the 0.5% concentration used for the autorosetting assays described in this thesis. The 0.5% FCS was routinely included in the autorosetting medium as it minimised thymocyte losses during handling.

2.10.2 Inhibition of Rosetting by Erythrocyte Sonicates

In this assay 50 µl of serial dilutions of erythrocyte sonicates were placed in the wells of microlitre plates. Preparation of sonicates is described in section 2.7.1. Usually the highest concentration of sonicates was prepared from packed (100%) erythrocytes. 25 µl of thymocytes (2×10^6/ml in medium) were then added to each well and the mixture incubated on ice for 60 min. The wells of the microlitre plate were filled with 200 µl of ice cold medium, the cells sedimented by centrifugation at 200 g for 1 min at 4°C and the supernatant discarded by flicking the plate. The cells in each well were washed once more with 200 µl of ice cold medium and then resuspended, by gentle vortexing, in 25 µl of ice cold medium. 25 µl of a 0.5% suspension of erythrocytes was added to each well and the standard rosetting assay then performed (see section 2.10).

2.10.3 Blocking Sonicate-Inhibition with Antisera

In some experiments erythrocytes were preincubated with

anti-H-2 sera prior to sonication and used in the inhibition assay. The preincubations were as follows: to 0.125 ml of packed erythrocytes was added 0.4 ml of either undiluted anti-H-2L^{d} serum or a 1/200 dilution of H-2.25 monoclonal antibody. The mixtures were left on ice for 30 min and the red cells then washed three times with medium prior to sonication as packed erythrocytes.

2.10.4 Inhibition of Rosetting by Sugars

This assay entailed incubating 25 µl of serial dilutions of the sugar with 25 µl of thymocytes (2 x 10^{6}/ml) for 1 hr on ice in the wells of microlitre plates. 50 µl of 0.5% erythrocytes suspension was then added to each well, and then the mixture pelleted and incubated on ice for 30-60 min. The number of rosetting cells was then determined as described in section 2.10.1

2.11 Autorosette Inhibition Factor (AIF)

2.11.1 Detection of AIF

Autorosette inhibition factor was detected by mixing 25 µl of a particular sample (usually serum or column fractions) with 25 µl of thymocytes (2 x 10^{6}/ml) and incubating the mixture on ice for 30 min. The thymocytes were then rosetted with 25 µl of 0.5% erythrocytes in the usual manner. To eliminate anti-red cell antibodies, sera from sheep, geese, chicken and bat were first absorbed with mouse erythrocytes (90 µl of neat serum + 10 µl of packed erythrocytes for 1 hr on ice before use).

For the absorption of AIF by thymocytes, 100 µl of 10% fresh BALB/c serum was preincubated with 100 µl of varying

number of BALB/c or C55 thymocytes for 45 min on ice. The mixture was spun at 400 g for 10 min at 4°C., and the supernatant used for inhibiting BALB/c thymocyte autorosetting.

2.11.2 Assaying Lymphoid Cells for AIF Secretion In Vitro

Cell suspensions from different lymphoid organs were washed twice with F15 medium lacking FCS but supplemented with 5×10^{-5} M 2-mercaptoethanol. This medium was used for all subsequent cell washing and culturing. Cells were made to a concentration of 2×10^{7}/ml in a total volume of 5 ml and incubated at 37°C for 6 hr in a 9 cm diameter plastic petri dishes in a humidified atmosphere of 5% CO_2 in air.

The supernatants of the cultures were harvested by pelleting the cells at 2000 g. The supernatants were then centrifuged at 30,000 g for 15 min at 4°C in a Sorvall rotor, concentrated 10-15 fold using vacuum dialysis, dialysed over-night at 4°C against PBS containing 1 mM EDTA and DTT, and assayed for AIF as described above.

Adherent peritoneal cells were prepared by incubating peritoneal cell suspensions in 5.5 cm plastic petri dishes at a concentration of $1x\ 10^{7}$ cells/ml in 2 ml of F15 medium containing 5% FCS at 37°C for 2 hr in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were collected, washed once and recultured in another dish for 6 hr at 1×10^{7} cells/ml. The adherent cell populations were washed three times with 5 ml of medium lacking FCS and then recultured for 6 hr in 2 ml medium. Culture supernatants were then collected and processed as described above.

2.11.3 Purification of AIF from Human Serum

Phosphocellulose purchased from Whatman Ltd., Kent, England was first precycled by the method of Greene et al (1978). The resin was stored until use in a storage buffer containing 0.5M sodium chloride, 1 mM EDTA, 1 mM DTT and 10 mM sodiun phosphate pH 7.4. After packing, the column was washed first with one column volume of storage buffer containing 50 mg/ml of bovine serum albumin (BSA) purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, England. The column was then washed with five column volumes of the storage buffer without BSA but with the sodium chloride concentration raised to 2.5M. The column was finally re-equilibrated with the normal storage buffer and used immediately.

All stages in the purification were carried out at 0°-4°C. Sixty ml of human blood was collected and immediately diluted into 200 ml of ice cold 0.15M sodium chloride, 0.17M sodium citrate solution containing 200 units of heparin. The citrated blood was then centrifuged at 2000 g for 20 min at 4°C. and the supernatant collected.

The diluted plasma was then dialysed overnight against 4 litres of the storage buffer. The dialysed plasma (251 ml) was then loaded onto a 3 x 20 cm column of precycled phospho-cellulose equilibrated in the same buffer. Under these conditions the bulk of the protein does not bind and passes through the ion-exchange column. The sodium chloride concentration in the buffer was increased to 0.8M and the column was washed with a further one litre of this buffer. The column was then eluted with a 500 ml linear salt gradient in which the sodium chloride concentration in the buffer was increased from 0.8M to 2.5M.

2.12 Haemagglutinin in Lymphoid Cell Lysates

2.12.1 Haemagglutination and Haemagglutination-Inhibition Assays

The haemagglutinating activity of lymphoid cell lysates prepared as described in section 2.7.2, was assessed in 96 well U-bottomed microlitre plates. 25 µl of the lymphocyte lysate was serially diluted using PBS containing 0.1% bovine serum albumin (BSA) as diluent. Then 25 µl of a 1% (v/v) erythrocyte suspension made up in the same diluent was added to each well, and haemagglutination was allowed to take place at 4°C on a flat surface. Haemagglutination titres were recorded at least 3 hr later.

The inhibitory activity of erythrocyte lysates was assessed by making 25 µl serial dilutions of the erythrocyte lysate in 0.1% BSA-PBS and adding 25 µl of a constant dilution of thymocyte lysate (haemagglutinin) to each well. After incubation on ice for 60 min, 25 µl of a 1% (v/v) suspension of erythrocytes was added to each well and haemagglutination titres assessed.

2.12.2 Sugar Inhibition of Haemagglutination

In this assay, 25 µl of sugar (at various concentrations) was added to each well of microtitre plates containing serial dilutions of the thymocyte lysate. The mixture was incubated on ice for 60 min, and then 25 µl of a 1% (v/v) suspension of erythrocytes added to each well and haemagglutination titres assessed 3 hr later.

2.12.3 Inhibition of Haemagglutination by AIF

In this assay, 25 µl of pure human AIF was serially diluted using PBS containing 0.1% BSA as diluent. Then 25 µl of a

thymocyte lysate at different dilutions was added to the wells containing AIF. The plate was incubated on ice for 30 min, 25 µl of a 1% (v/v) suspension of erythrocytes was added to each well and haemagglutination titres assessed.

The human AIF in this inhibition assay was purified from human plasma as described in section 2.11.3.

2.13 Isolation of Autorosetting Receptors and Acceptors

Figure 2.1 depicts the experimental protocol used to purify and characterise the thymocyte haemagglutinin. The protocol consisted of binding the haemagglutinin in a ^{125}I-labelled lysate to glutaraldehyde fixed red cells, eluting the haemagglutinin from the red cells with D-galactosamine (20mg/ml) and analysing the eluate by SDS-PAGE. In all experiments a control was included where the red cells were eluted with D-glucose (20 mg/ml) rather than D-galactosamine. Furthermore, a fraction of each eluate was depleted of sugar by dialysing and tested for the recovery of haemagglutinin acceptor from ^{125}I-labelled mouse erythrocytes, except that the erythrocyte lysate was bound to and eluted from thymocytes and recovery of haemagglutination-inhibition activity was determined.

2.14 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.14.1 SDS-PAGE of Serum Proteins

Different serum fractions and AIF samples were analysed by gradient SDS-PAGE performed essentailly as described by Laemmli et al (1970). Reduction of the sample was performed with 10% 2-mercaptoethanol in 1% SDS in 50 mM Tris pH 6.8 buffer in a

Fig. 2.1

^{125}I-labelled Thymocytes
(lactoperoxidase catalysed iodination)

↓

Solubilisation in 0.5% NP-40 PBS containing PMSF. Spin out nuclei and debris (28,000 g, 15 min)

↓

Absorb lysate to glutaraldehyde fixed mouse red cells, 2½ hr., 4°C.

↓

Wash red cells X 2 with 0.01% NP-40-PBS/0.1% BSA

↓

Elute receptor from red cells with 20 mg/ml of D-galactosamine in 0.01% NP-40 PBS, 1½ hr., 4°C.

↓

Collect supernatant

↙ ↘

Analyse on SDS-PAGE

Dialyse against 0.01% NP-40 PBS, 4 hr., 4°C.

↓

Test for haemagglutination

boiling water bath for 1.5 min and electrophoresis carried out on a gradient of 10-20% polyacrylamide for 16 hr at 50V. The gel was stained with 0.25% Coomassie Blue R-250 in 10% acetic acid and 50% methanol (v/v) at 37°C for 1 hr.

2.14.2 SDS-PAGE of Autorosetting Receptors and Acceptors

^{125}I-labelled material eluted from glutaraldehyde fixed erythrocytes or thymocytes was analysed using SDS-PAGE essentailly as described by Maizel (1971). Gels used consisted of a 10.6 cm resolving gel and 2.5 cm of stacking gel of 0.6 cm in diameter. Final concentrations in the resolving gel were: 10% acrylamide (Cyanamid, Australia), 0.266% N, N'-bis-methylene acrylamide (bis acrylamide, Eastman Chemicals Co); 0.375M Tris (Sigma) HCl, pH 8.9; and 0.1% SDS (Sigma). Polymerisation was catalysed with N, N, N'-tetra-methylethylenediamine (TEMED, Eastman Chemical Co.), final concentration 3.33 mM, and ammonium persulphate (BDH), final concentration 2.2 mM. Stacking gels contained 3% acrylamide, 0.08% bis-acrylamide, 0.0625M Tris/HCl, pH 7.6, and 0.1% SDS. Stacking gels were polymerised with 3.33mM TEMED and 4.4 mM ammonium persulphate. Prior to use, SDS was recrystallised from ethanol, acrylamide from chloroform, and bis-acrylamide from acetone. The electrode buffer contained 0.384 mM glycine, 0.0495 M Tris, and 0.1% SDS, and was the same for both upper and lower buffer compartments. Samples for SDS-PAGE were dissolved by boiling for 1-3 min in appropriate volumes of a solution containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 0.0625 M Tris-HCl. The pH of the dissolving mix was adjusted to approximately 7.5 with 1N NaOH.

Electrophoresis was carried out at 60 watts until the dye marker entered the resolving gel and then run at 120 volts. Gels were cut into 2 mm fractions using a gel slicer, and counted in a Packard Auto-gamma Counter.

Chapter 3

Evidence that the Autorosetting Phenomenon is under MHC Control

Table of Contents

3.1 Introduction

There have been numerous reports in the past that substantial subpopulations of murine lymphocytes can form rosettes with both autologous and allogeneic erythrocytes (Micklem and Asfi 1971, Sandilands *et al* 1974, Charriere and Bach 1975, Braganza *et al* 1975, Kolb 1977). However, the nature of this interaction and its functional significance has remained a mystery (see chapter 1).

In recent years it has become increasing apparent that products of the major histocompatibility complex (MHC) of the mice and other species play a critical role in immune induction. For example, self H-2K and H-2L/H-2D antigens play a vital part in the recognition of foreign antigens by cytotoxic T cells (Doherty *et al* 1976, Shearer *et al* 1976). In a similar manner, it appears that self structures mapping to the I-region are recognized in association with foreign antigen by the T cells which express help (Katz and Benacerraf 1975), delayed-type hypersensitivity (Miller *et al* 1976) and antigen-specfic proliferation *in vitro* (Thomas *et al* 1977, Schwartz *et al* 1978). Although the mechanism of recognition of self plus foreign antigen in these systems is still uncertain frequently it has been suggested that two receptors are involved, one recognizing foreign antigen, the other recognizing a self MHC product (Janeway *et al* 1976, Zinkernagel *et al* 1978).

With these concepts in mind, it appeared possible that the interaction between lymphocytes and autologous erythrocytes might be mediated by an anti-self receptor on lymphocytes which had syngeneic preference for a MHC gene product on erythrocytes. This anti-self receptor would probably weakly cross react with other haplotypes and thus multipoint binding via a low affinity receptor, would explain the interaction of lymphocytes with both allogeneic and syngeneic

erythrocytes.

This chapter describes the experimental procedures undertaken to test this hypothesis. The experimental protocol was to measure the ability of different erythrocyte sonicates to inhibit the rosetting of thymocytes and peripheral lymphocytes of different inbred strains of mice with autologous red cells. Using this method it was possible to demonstrate that autorosette formation is, indeed, mediated by an H-2 controlled receptor. In fact, the receptor on murine thymocytes and peripheral T and B lymphocytes appeared to be primarily directed against an H-2L region controlled molecule. Furthermore, with the recent demonstration that the H-2 antigens on the thymic epithelium determine the specificity of H-2 restricted cytotoxic T cells (Bevan 1977, Zinkernagel et al 1978), the influence of the thymic environment on the specificity of H-2L region controlled rosetting was examined. In addition, the specificity of the autorosetting receptors in F1 hybrid mice was analysed.

3.2 Results

3.2.1 Efficiency of Autorosetting

Table 3.1 lists the proportion of rosetting cells in the thymus, spleen, lymph node and bone marrow of seven strains of mice. Substantial subpopulations autorosette in each lymphoid organ, the autorosette frequency being thymus > spleen > lymph node > bone marrow. An additional important feature of Table 3.1 is that the lymphoid organs of athymic BALB/c nude mice contained a slightly higher proportion of autorosetting cells than normal BALB/c mice. This result implies that thymus-independent cells can autorosette.

Initially when the autorosetting of lymphoid cells was examined it became apparent, however, that mouse strains varied in their

Table 3.1

Proportion of Autorosetting Cells in Different Lymphoid Organs

Mouse Strain	Haplotype	Lymphoid Oran			
		Thymus	Spleen	Lymph Node	Bone Marrow
B10.BR	k	59 ± 3.1 [a)]	26 ± 2.5	16 ± 1.0	13 ± 1.2
BALB/c	d	43 ± 2.3	14 ± 2.4	13 ± 0.7	6 ± 1.5
BALB/c (nude)	d	-	20 ± 2.5	1.5 ± 1.0	11 ± 1.0
DBA/2	d	38 ± 2.8	20 ± 3.5	10 ± 1.4	8 ± 1.4
A.SW	s	58 ± 4.2	18 ± 2.6	14 ± 2.0	10 ± 2.0
B10.G	q	58 ± 2.9	26 ± 2.7	12 ± 1.5	10 ± 2.0
B10	b	54 ± 1.2	13 ± 1.2	8 ± 1.4	7 ± 0.7

a) Results expressed as % rosette forming cells ± standard deviation of at least three determinations. Rosetting performed between autologous lymphocytes and erythrocytes in each case.

autorosetting potential. For example, only 5% of SJL/J spleen cells formed autorosettes, whereas 26% of B10.BR splenocytes autorosetted. By rosetting different combinations of lymphocytes and erythrocytes, it was found that the source of variation usually resided in the rosetting erythrocyte. Table 3.2 presents three strain combinations which clearly demonstrate this point. A higher proportion (26%) of B10.BR spleen cells rosetted with B10.BR red cells, whereas a much lower proportion (8%) of CBA/H spleen cells formed autorosettes. However, the converse result was observed when B10.BR spleen cells were rosetted with CBA/H red cells or CBA/H splenocytes were rosetted with B10.BR erythrocytes. Similar results were obtained when the autologous and heterologous rosetting of two other strain combinations were compared, namely A.SW with SJL/J and DBA/1 with B10.G (Table 3.2). The same trends were observed when different combinations of thymocytes and erythrocytes were rosetted, although the combinations were less marked than with spleen cells (Table 3.2). In contrast, there was little difference between autologous and heterologous rosetting when lymph node and bone marrow cells were examined (see Table 3.8).

Since H-2 compatible strains were compared in Table 3.2, background (non H-2) genes must influence the ability of erythrocytes to bind to lymphocytes. One possibility is that these background genes modify rosetting efficiency by varying the density of H-2 molecules on the red cell surface. This seems unlikely as allo-antibody absorption studies demonstrated that B10.BR erythrocytes, which rosetted very well, express approximately eight times less H-2 antigen than CBA/H red cells, which rosette poorly. A more likely explanation is that after specific union between lymphocyte and erythrocyte the complex is stabilized by non-specific interactions

Table 3.2

Influence of Erythrocyte Background on Rosetting Efficiency of Spleen Cells and Thymocytes

Lymphocyte Source	Erythrocyte Source	Rosetting Lymphocytes	
		Spleen	Thymus
		a)	
B10.BR(H-2^k)	B10.BR	26 ± 2.5	57 ± 3.2
B10.BR	CBA/H	10 ± 2.1	34 ± 2.8
CBA/H(H-2^k)	CBA/H	8 ± 1.4	34 ± 2.3
CBA/H	B10.BR	28 ± 2.1	60 ± 3.5
A.SW(H-2^s)	A.SW	18 ± 2.6	58 ± 3.5
A.SW	SJL/J	3 ± 0.6	30 ± 2.1
SJL/J(H-2^s)	A.SW	20 ± 1.5	58 ± 2.8
DBA/1(H-2^q)	DBA/1	12 ± 0.6	42 ± 3.0
DBA/1	B10.G	26 ± 2.0	49 ± 4.2
B10.G(H-2^q)	B10.G	26 ± 2.7	58 ± 2.8
B10.G	DBA/1	12 ± 1.0	44 ± 2.8

a) Results expressed as % rosette forming cells ± standard deviation of at least three determinations.

(e.g. electrostatic forces) between the red cell and lymphocyte membranes. Obviously these non-specific forces which stabilize the rosettes are not under H-2 control.

Table 3.2 presents selected examples of the effect of the erythrocyte background on the rosetting between thymocytes and erythrocytes from nine different strains which have been performed, i.e., B10.BR, CBA/H, BALB/c, DBA/2, A.SW, SJL/J, DBA/1, B10.G and C57BL/6. Of the 81 combinations tested, any variations in rosetting efficiency can be attributed to the red cell background in all except three cases, namely the rosetting of SJL/J thymocytes with either BALB/c or DBA/2 red cells and the rosetting of DBA/1 thymocytes with C57BL/6 erythrocytes. In these three instances the proportion of rosetting thymocytes was lower than expected. Similar checker-board analysis of the rosetting of spleen cells, although incomplete, has given similar results to thymocytes.

The rosette assay used has detected a higher frequency of autorosettes in lymphoid organs than reported by some groups (Micklem and Asfi 1971, Charrier and Bach 1975, Braganza et al 1975, Nabarra and Charriere 1979, Primi et al 1979) but has given similar results to other investigators (Kolb 1977, Sandilands et al 1974). The reasons for these differences has been discussed earlier (chapter 1 & 2). It should be re-emphasized, however, that the largest sources of error are the inhibitory effects of serum (Kolb 1977, Parish et al 1974, Kolb 1978) and the mechanical disruption of rosettes during their resuspension, i.e., resuspension of rosettes on a roller should be avoided.

3.2.2 Inhibition Assay for Assessing Specificity of Autorosetting

It is well establish that subpopulations of murine lymphocytes form rosettes with both syngeneic and allogeneic erythrocytes (Micklem and Asfi 1971, Sandilands et al 1974, Charriere and Bach 1975,

Braganza et al 1975, Kolb 1977). However, this rosetting phenomenon may be due to a strain-specific receptor whose specificity is masked by multi-point interaction between lymphocytes and erythrocytes. In order to test this hypothesis an inhibition assay was developed which entailed pre-incubating the lymphoid cells with different erythrocyte sonicates, washing the lymphocytes and then rosetting the lymphocytes with autologous erythrocytes. Using this assay it was, indeed, possible to demonstrate some strain specificity in both the thymocyte and spleen cell receptors which recognize autologous erythrocytes. It must be emphasized that to increase the sensitivity of sonicate inhibition the lowest lymphocyte: red cell ratio required for maximum autorosetting was used (see section 2.10.1).

Table 3.3 summaries the results obtained with this inhibition assay when thymocytes from seven different mouse strains were tested, It was found that with C57BL/6, SJL/J and DBA/1 thymocytes the auto-rosette inhibition was completely strain specific. In contrast, inhibition was strongly cross-reactive between BALB/c, DBA/2, CBA/H and B10.BR mouse strains. This result suggested that in BALB/c, DBA/2, CBA/H and B10.BR mice the thymocyte receptors have identical or very similar specificity. The complete inhibition data obtained with BALB/c thymocytes is depicted in Fig. 3.1. Again it can be seen that BALB/c DBA/2, CBA/H and B10.BR erythrocyte sonicates show strong inhibition whereas C57BL/6, SJL/J and DBA/1 sonicates are not inhibitory.

Subsequently, spleen cell autorosette inhibition from six mouse strains and BALB/c (nude) mouse was also found to be completely strain specific (Tables 3.4 & 3.5). Again, inhibition of autorosetting of these lymphoid cells was strongly cross-reactive between the ($\underline{H\text{-}2}^{d}$) and ($\underline{H\text{-}2}^{k}$) haplotypes.

Table 3.3

Strain Specificity of Autorosette Inhibition

Thymocytes	Erythrocytes	Erythrocyte Sonicate[a]							
		Nil	BALB/c ($H\text{-}2^d$)	DBA/2 ($H\text{-}2^d$)	CBA/H ($H\text{-}2^k$)	B10.BR ($H\text{-}2^k$)	C57BL/6 ($H\text{-}2^b$)	SJL/J ($H\text{-}2^s$)	DBA/1 ($H\text{-}2^q$)
BALB/c	BALB/c	43±2.5	[9±3.0][b]	[5±2.0]	[11±2.1]	[17±1.4]	43±2.1	40±2.5	44±2.0
DBA/2	DBA/2	33±1.5	[5±2.0]	[4±2.0]	[5±2.5]	[13±1.5]	30±1.6	31±2.1	31±2.1
CBA/H	CBA/H	34±2.3	[6±2.5]	[6±2.0]	[6±1.2]	[9±2.1]	31±2.1	32±2.0	31±1.5
B10.BR	B10.BR	57±3.2	[26±2.1]	[24±2.1]	[19±3.5]	[29±0.7]	55±3.5	57±3.5	57±0.7
C57BL/6	C57BL/6	45±2.7	44±2.1	45±1.5	45±2.1	43±3.1	[14±2.0]	43±4.0	44±2.5
SJL/J	SJL/J	21±1.6	20±2.4	22±1.5	21±3.1	21±2.0	21±2.6	[5±1.0]	20±1.5
DBA/1	DBA/1	42±3.0	41±2.1	43±2.6	40±1.5	41±1.7	42±2.1	43±3.1	[5±3.5]

a) Thymocytes preincubated for 60 min at 4°C with 100% erythrocyte sonicate prior to rosetting with autologous erythrocytes.

b) Results expressed as % rosetting thymocytes ± standard deviation of mean of three determinations. Values which represent significant inhibition are enclosed.

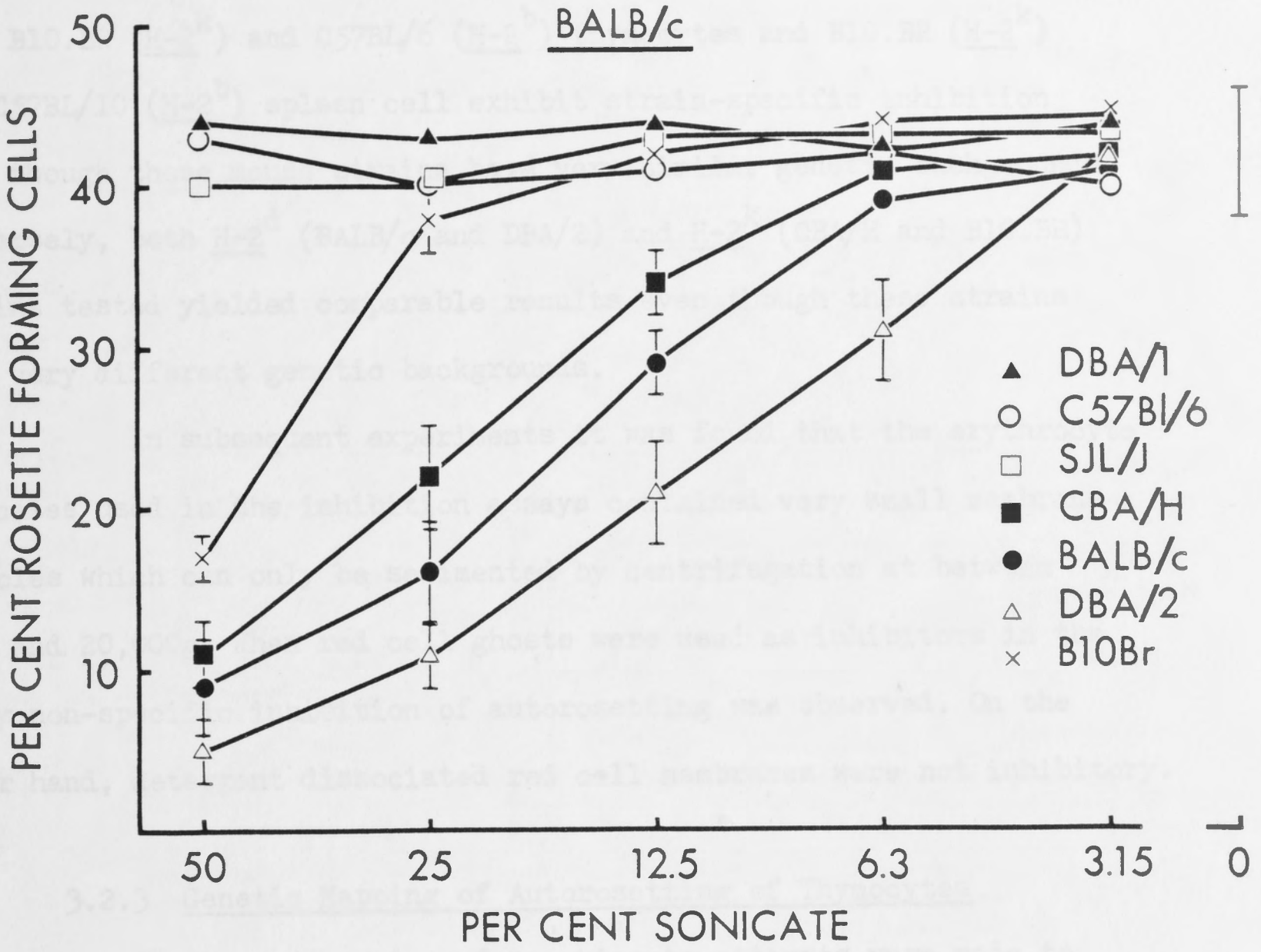

Fig. 3.1 Ability of erythrocyte sonicates from different mouse strains to inhibit the rosetting of BALB/c thymocytes with BALB/c erythrocytes. The erythrocyte sonicates used as inhibitors are listed on the figure. Thymocytes were preincubated with different concentrations of sonicates prior to rosetting. Vertical bars represent standard deviations of means. The rosetting of control thymocytes, which were not preincubated with sonicates, is indicated in the right hand margin of the figure.

It should be noted that the data in Tables 3.3 -3.5 and Fig. 3.1 tend to implicate the H-2 complex in autorosette-inhibition. Thus B10.BR ($H\text{-}2^k$) and C57BL/6 ($H\text{-}2^b$) thymocytes and B10.BR ($H\text{-}2^k$) and C57BL/10 ($H\text{-}2^b$) spleen cell exhibit strain-specific inhibition even though these mouse strains have very similar genetic backgrounds. Conversely, both $H\text{-}2^d$ (BALB/c and DBA/2) and $H\text{-}2^k$ (CBA/H and B10.BR) strains tested yielded comparable results even though these strains have very different genetic backgrounds.

In subsequent experiments it was found that the erythrocyte sonicates used in the inhibition assays contained very small membrane vesicles which can only be sedimented by centrifugation at between 5000 and 20,000g. When red cell ghosts were used as inhibitors in the assay non-specific inhibition of autorosetting was observed. On the other hand, detergent dissociated red cell membranes were not inhibitory.

3.2.3 Genetic Mapping of Autorosetting of Thymocytes

In the next series of experiments attempts were made to map the gene(s) responsible for the strain specific inhibition of autorosetting. Figs. 3.2 and 3.3 depict the complete inhibition data of the thymocytes obtained with the seven strains examined in Table 3.3. Virtually identical inhibition data was obtained with BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), CBA/H ($H\text{-}2^k$) and B10.BR ($H\text{-}2^k$) thymocytes (Fig. 3.2). Inhibition of rosetting was only obtained with sonicates from mice expressed either the d (i.e., BALB/c, DBA/2, A.TH) or k (i.e., B10.BR, CBA/H) haplotype in the H-2L/H-2D region of the MHC. However, the most striking result was that the erythrocyte sonicate from the BALB/c-$H\text{-}2^{dm2}$ mutant mouse (C55) strain was not inhibitory. This strain carries an $H\text{-}2L^d$ deletion mutation (McKenzie et al 1977, Hansen et al 1977, Demant and Neauport-Sautes 1978) and, therefore, this finding

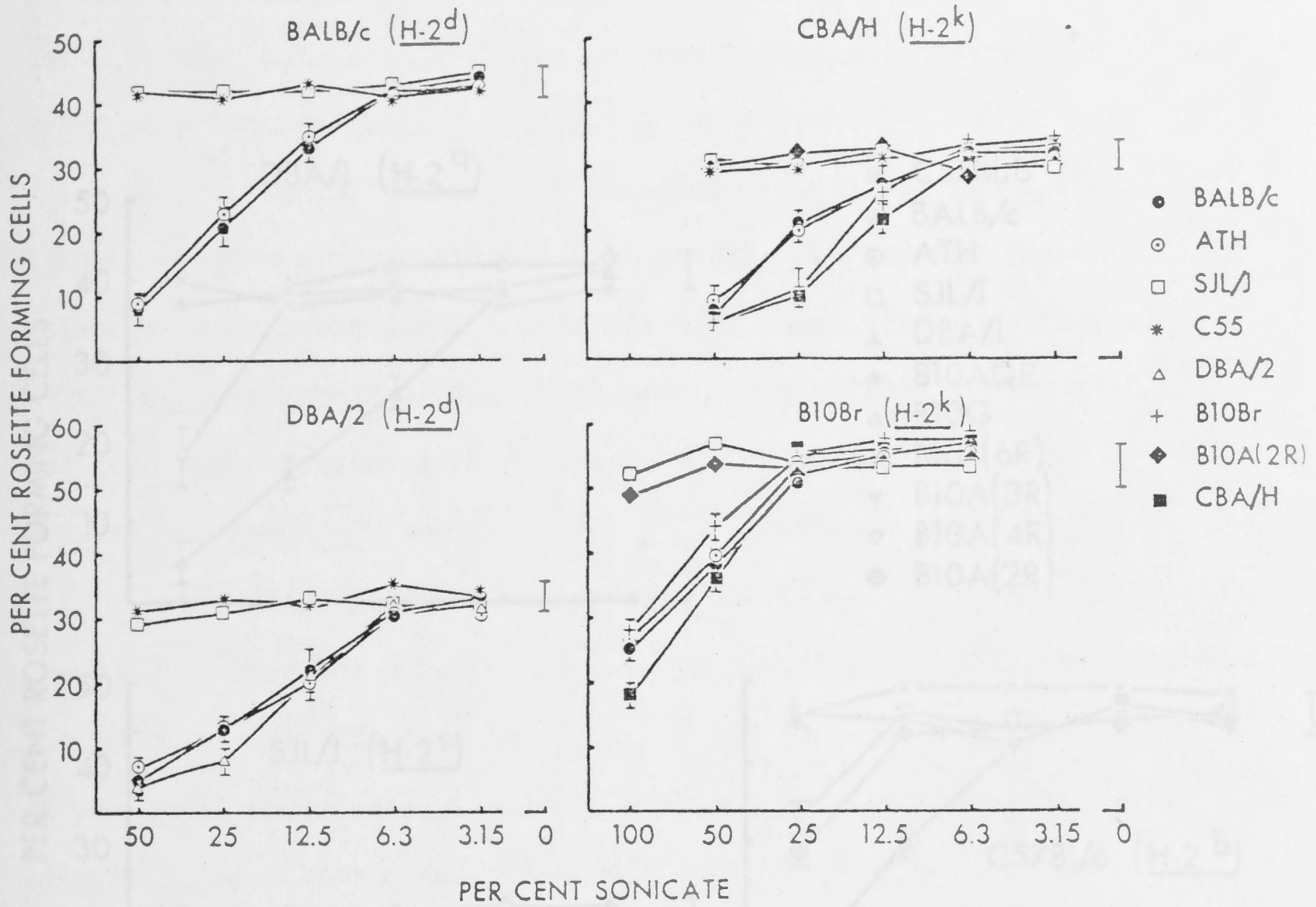

Fig. 3.2 Genetic mapping of inhibition of thymocyte autorosetting by erythrocyte sonicates. Each graph is headed with the strain and H-2 haplotype of the thymocytes considered. The erythrocyte sonicates used as inhibitors are listed on the right hand side of the Figure. Vertical bars represent standard deviations of means. The autorosetting of thymocytes, in the absence of inhibitors, is indicated in the right hand margin of each graph.

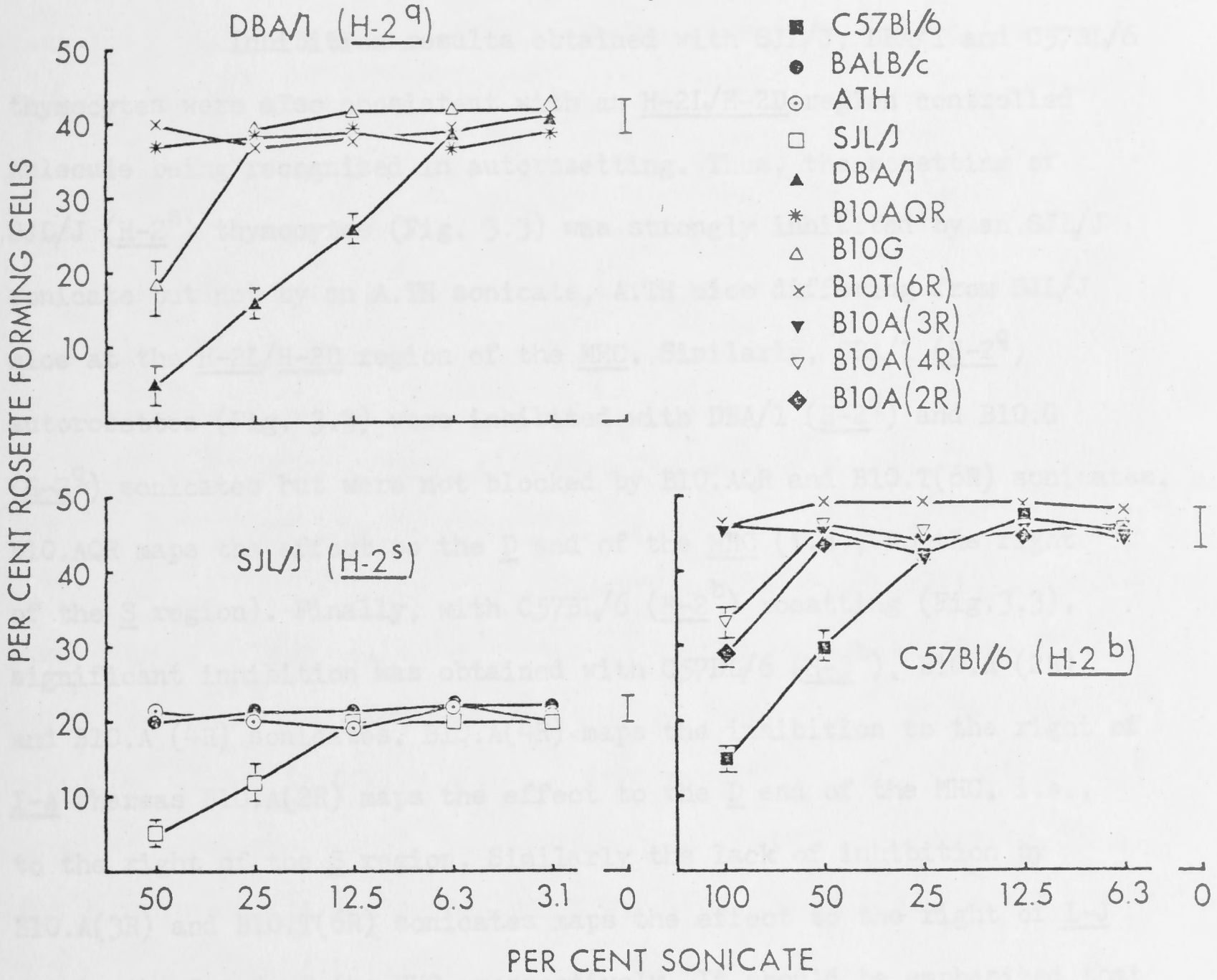

Fig. 3.3 Genetic mapping of inhibition of thymocyte autorosetting by erythrocyte sonicates.

Legends as in Fig. 3.2

strongly suggests that BALB/c, DBA/2, CBA/H and B10.BR thymocytes are recognizing an H-2L region controlled structure on mouse erythrocytes. Further analysis of this mutant strain will be presented later in this chapter (section 3.2.5). An additional implication of these results is that, in this autorosetting test, thymocytes cannot distinguish between the H-2L molecules carried by d and k haplotypes.

Inhibition results obtained with SJL/J, DBA/1 and C57BL/6 thymocytes were also consistent with an H-2L/H-2D region controlled molecule being recognized in autorosetting. Thus, the rosetting of SJL/J ($H\text{-}2^s$) thymocytes (Fig. 3.3) was strongly inhibited by an SJL/J sonicate but not by an A.TH sonicate, A.TH mice differing from SJL/J mice at the H-2L/H-2D region of the MHC. Similarly, DBA/1 ($H\text{-}2^q$) autorosettes (Fig. 3.3) were inhibited with DBA/1 ($H\text{-}2^q$) and B10.G ($H\text{-}2^q$) sonicates but were not blocked by B10.AQR and B10.T(6R) sonicates. B10.AQR maps the effect to the D end of the MHC (i.e., to the right of the S region). Finally, with C57BL/6 ($H\text{-}2^b$) rosetting (Fig.3.3), significant inhibition was obtained with C57BL/6 ($H\text{-}2^b$), B10.A (2R) and B10.A (4R) sonicates. B10.A(4R) maps the inhibition to the right of I-A whereas B10.A(2R) maps the effect to the D end of the MHC, i.e., to the right of the S region. Similarly the lack of inhibition by B10.A(3R) and B10.T(6R) sonicates maps the effect to the right of I-J and to the D end of the MHC, respectively. It should be emphasized that the inhibition of C57BL/6 autorosetting was not as marked as with other strains. However, the inhibition obtained with the 100% sonicates of B10.A(2R) and B10.A(4R) erythrocytes was statistically significant ($p<0.05$).

The autorosette-inhibition of two additional mouse strains has also been genetically mapped (data not shown), namely the B10.G ($H\text{-}2^q$) and A.SW ($H\text{-}2^s$) strains. Inhibition of B10.G rosetting followed

the same genetics as that obtained with DBA/1 (H-2^q) thymocytes (Fig. 3.3). Similarly, A.SW autorosette-inhibition corresponded to the genetics observed with H-2 compatible SJL/J thymocytes (Fig. 3.3).

During these inhibition studies it became apparent that erythrocyte sonicates from mouse strains carrying the B10 background tended to be substantially less inhibitory than sonicates from other strains, e.g., CBA/H compared with B10.BR in Fig. 3.1 and Table 3.2, DBA/1 compared with B10.G in Fig. 3.3 and C57BL/6 compared with B10 recombinants in Fig. 3.3. This effect may reflect a lower density of H-2 molecules on the surface of erythrocytes from mice with the B10 background. Preliminary alloantibody absorption studies are consistent with this interpretation.

3.2.4 Genetic Mapping of Autorosetting of Spleen, Lymph Node and Bone Marrow Cells

The ability of erythrocyte sonicate to inhibit the autorosetting of spleen cells from seven mouse strains was assessed. The inhibition assay was identical to that described above for thymocytes (section 3.2.3). Initially the haplotype specificity of inhibition was examined (data not shown). It was found that, as with thymocytes, b, q and s haplotypes exhibited haplotype specific inhibition, whereas d and k haplotypes showed cross-reactive inhibition.

By the use of recombinant mouse strains it was, subsequently, possible to map the autorosette-inhibition within the H-2 complex. With B10.BR (H-2^k), BALB/c (H-2^d), BALB/c nude (H-2^d) and DBA/2 (H-2^d) spleen cells,inhibition of autorosetting only occurred when the erythrocyte sonicates carried either the d or k haplotype in the H-2L/H-2D region of the MHC (Table 3.3). Furthermore, erythrocyte sonicates from the H-2L deletion mutant (BALB/c-H-2^{dm2}) or (C55) strain lacked inhibitory activity, which mapped autorosette-inhibition to the H-2L region. The complete inhibition data for DBA/2, B10.BR

and BALB/c strains is depicted in Fig. 3.4.

Similar inhibition experiments with A.SW (H-2^s) spleen cells mapped inhibition to the H-2L/H-2D region, i.e., A.TH sonicates were not inhibitory (Table 3.4). In a similar manner, the inhibition of B10.G (H-2^q) and B10 (H-2^b) autorosettes was mapped to the D-end of the MHC, i.e., to the right of the S region (Table 3.5). The key recombinant strains for the genetic mapping of B10.G inhibition were B10.T(6R) and B10. AQR, whereas B10 inhibition was mapped by the B10.A(2R) and B10. A(4R) recombinant strains.

The specificity of the autorosetting receptor on lymph node and bone marrow cells was also examined using the inhibition assay. The results obtained with B10.BR lymphoid cells are presented in Table 3.6. As with spleen cells, autorosette-inhibition was mapped to the H-2L/H-2D region for the lymph node and bone marrow. In this experiment subpopulations of spleen cells were also examined for autorosetting potential. The Ig$^-$ (B cell depleted) population of spleen could still autorosette but not as effectively as unfractionated or Thy-1.2$^-$(T cell depleted) spleen. The proportion of autorosettes in Thy-1.2$^-$spleen was consistent with the results obtained with T cell depleted (nude) mice (Tables 3.1 & 3.4). However, the recovery of autorosetting cells in Thy-1.2$^-$ spleen was slightly lower than expected. It was subsequently shown that this loss was due to the blocking effects of guinea pig complement (Kolb 1977 and 1978). When B10.BR spleen was depleted of both Ig$^+$ (B) and Thy-1.2$^+$ (T) cells by a double resetting procedure (see chapter 2, section 2.6.2) the remaining population of null cells was virtually unable to form rosettes (approx. 1%). From these data it can be calculated that approximately 80% of the rosetting spleen cells are B lymphocytes and 20% T lymphocytes. Calculated in another way, if one assumes 60% B cells and 30% T cells in B10.BR spleen, approximately 30% of B cells and 15% of T cells can rosette with autologous erythrocytes. Furthermore,

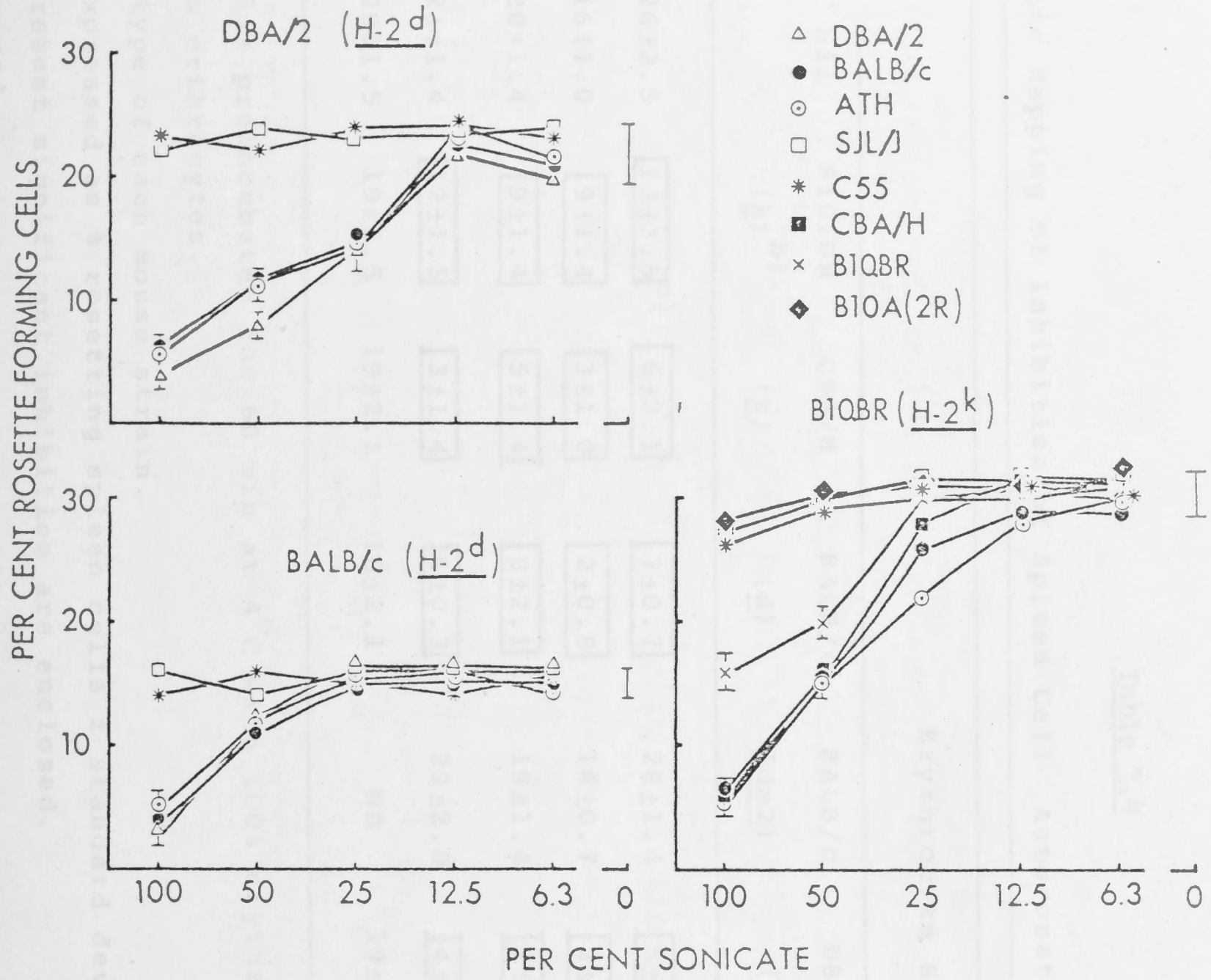

Fig. 3.4 Genetic mapping of inhibition of spleen cell autorosetting by erythrocyte sonicates. Each graph is headed with the strain and H-2 haplotype of the spleen cells considered. The erythrocyte sonicates used as inhibitors are listed on the right hand side of the figure. Vertical bars represent standard deviations of means. The autorosetting of spleen cells, in absence of the inhibitors, is indicated in the right hand margin of each graph.

Table 3.4

Genetic Mapping of Inhibition of Spleen Cell Autorosetting: H-2^k, H-2^d, and H-2^s Haplotypes

Autorosetting Strain		Erythrocyte Sonicate[a]									
		Nil	B10.BR (k)[b]	CBA/H (k)	BALB/c (d)	BALB/c (dm2)	DBA/2 (d)	A.SW (s)	SJL/J (s)	A.TH (t2)	B10.A(2R) (h2)
B10.BR	(k)	26±2.5	[13±3.5][c]	[6±2.1]	[7±0.7]	28±1.4	[5±1.4]	28±2.1	28±1.5	[6±2.3]	28±1.5
BALB/c	(d)	16±1.0	[9±1.4]	[3±1.0]	[2±0.8]	14±0.7	[3±2.1]	15±1.0	14±1.1	[5±1.4]	16±0.7
BALB/c (nude)	(d)	20±1.4	[9±1.4]	[5±1.4]	[8±2.1]	19±1.4	[7±2.1]	20±2.1	22±1.4	[3±1.4]	19±2.2
DBA/2	(d)	21±1.4	[12±1.5]	[3±1.4]	[6±0.7]	22±2.8	[4±0.6]	21±2.8	19±0.7	[5±2.8]	22±2.8
A.SW	(s)	20±1.5	19±3.5	19±2.1	18±2.1	ND	19±2.1	[4±0.6]	[10±2.8]	20±0.6	ND

a) Spleen Cells preincubated for 60 min at 4°C with 100% erythrocyte sonicate prior to rosetting with autologous erthrocytes.

b) H-2 haplotype of each mouse strain.

c) Results expressed as % rosetting spleen cells ± standard deviation of three determinations. Values which represent significant inhibition are enclosed.

ND Not determined.

Table 3.5

Genetic Mapping of Inhibition of Spleen Cell Autorosetting: H-2^b and H-2^q Haplotypes

Autorosetting Strain	Erythrocyte Sonicate [a]								
	Nil	B10.G (q) [b]	B10 (b)	B10.BR (k)	B10.T(6R) (y2)	B10.AQR (y1)	B10.A(2R) (h2)	B10.A(3R) (i3)	B10.A(4R) (h4)
B10.G (q)	26 ± 2.7	11 ± 2.1 [c]	29 ± 0.7	30 ± 2.0	25 ± 1.4	26 ± 1.4	ND	ND	ND
B10 (b)	13 ± 1.2	13 ± 0.7	6 ± 2.0	15 ± 0.7	ND	ND	2 ± 1.1	14 ± 0.7	4 ± 0.7

Footnotes as in Table 3.4

Table 3.6

Genetic Mapping of Spleen, Lymph Node and Bone Marrow Autorosetting in B10.BR (H-2^k) Mice [a]

Lymphoid Organ	Erythrocyte Sonicate						
	Nil	B10.BR (k) [b]	CBA/H (k)	BALB/c (d)	SJL/J (s)	A.TH (t2)	B10.A(2R) (h2)
Spleen	26 ± 2.5	13 ± 3.5 [c]	6 ± 2.1	7 ± 1.5	28 ± 1.4	6 ± 2.3	28 ± 2.8
Ig^- Spleen	12 ± 1.5	4 ± 0.7	1 ± 0.7	1 ± 1.0	12 ± 0.7	1 ± 0.7	13 ± 1.4
Thy-1.2^- Spleen	22 ± 1.7	11 ± 0.7	5 ± 0.7	1 ± 2.0	20 ± 1.4	3 ± 2.1	21 ± 2.8
Lymph Node	16 ± 0.7	5 ± 1.4	3 ± 1.4	5 ± 0.7	15 ± 0.7	6 ± 1.5	15 ± 1.0
Bone Marrow	14 ± 1.5	7 ± 1.5	4 ± 1.0	2 ± 1.0	13 ± 1.0	3 ± 1.5	14 ± 0.7

a) Autorosetting is inhibited with different erythrocyte sonicates.

Footnotes as in Table 3.4

genetic mapping studies using the inhibition assay, revealed that both B (Thy-1.2$^-$)and T (Ig$^-$) lymphocytes autorosetted via H-2L/H-2D region restricted receptors (Table 3.6).

The inhibition studies with spleen cells confirmed the observation with thymocytes described above (section 3.2.3) that erythrocyte sonicates from mouse strains carrying the B10 background are less inhibitory than sonicates from other strains e.g., compare B10.BR and CBA/H sonicates in Fig. 3.4 and Table 3.4. This difference is probably due to the lower density of H-2 molecules carried by erythrocytes from B10 background mice (see section 3.2.1 and Table 3.2). Thus erythrocyte sonicates behave very differently from intact red cells as non-H-2 structures, rather than H-2 density, influence the ability of whole erythrocytes to bind to lymphocytes (Table 3.2).

3.2.5 Analysis of Autorosetting in H-2L/H-2D Region Mutant Mice

Experiments described earlier in this chapter demonstrated that erythrocyte sonicates from BALB/c-H-2^{dm2} mutant mice were unable to inhibit the autorosetting of BALB/c thymocytes (Fig. 3.2) and spleen cells (Fig. 3.4). Lymphoid cells of the BALB/c-H-2^{dm2} mutant mice to rosette with autologous, allogeneic or xenogeneic red cells were then tested. The mutant thymocytes were almost devoid of rosetting activity (1 - 3%) with all erythrocytes tested (Table 3.7). In addition, the mutant spleen, lymph node and bone marrow cells were also found to rosette less effectively (Table 3.8). Mutant lymph node, however, rosetted with mutant red cells (7%) almost as effectively as wild type lymph node (11%). The specificity of the receptor on the residual population of rosetting cells in the BALB/c-H-2^{dm2} mutant is unknown. Attempts to inhibit these rosetting cells with erythrocyte sonicates have, so far, failed. In contrast, the lymphoid cells of the wild-type BALB/c strain strongly rosetted with all the target erythrocytes used (18% - 55%).

Table 3.7

Rosetting of BALB/c Mutant (H-2^{dm2}) [a] and BALB/c(H-2^{d}) Thymocytes with Different Erythrocytes

Thymocyte	Rosetting Erythrocyte									
	BALB/c (H-2^{dm2})	BALB/c (H-2^{d})	DBA/2 (H-2^{d})	B10.BR (H-2^{k})	CBA/H (H-2^{k})	C57BL/6 (H-2^{b})	SJL/J (H-2^{s})	DBA/1 (H-2^{q})	B10.G (H-2^{q})	Lewis Rat
BALB/c(H-2^{dm2})	1±1.7 [b]	2±2	2±2	2±2	1±1	2±1	1±1	2±0.6	2±1.5	3±1.4
BALB/c(H-2^{d})	48±4.2	40±2.1	36±2.1	55±3.5	32±3.5	47±2.1	27±4.2	46±2.8	53±2.1	18±1.4

a) BALB/c-H-2^{dm2} strain also designated as C55 strain

b) Results expressed as % rosetting thymocytes ± standard deviation of at least three determinations.

Table 3.8

Rosetting of Lymphoid Organs from Mutant (H-2^{dm2}) and Wild-type (H-2^{d}) BALB/c mice with Different Erythrocytes

BALB/c Strain Rosetted	Lymphoid Organ Rosetted	Rosetting Erythrocyte							
		BALB/c mutant (dm2) [a]	BALB/c wild-type (d)	DBA/2 (d)	B10.BR (k)	A.SW (s)	B10.G (q)	B10 (b)	Lewis rat
Mutant	Spleen	1±0.8 [b]	3±0.7	3±1.4	3±1.4	2±0.7	4±1.7	2±1.3	3±1.2
Wild type	Spleen	16±1.4	14±0.7	22±2.1	28±1.4	20±2.1	30±1.4	13±1.4	9±1.4
Mutant	Lymph node	7±1.5	3±0.7	2±0.7	4±1.1	3±0.7	3±1.2	2±1.0	2±0.7
Wild type	Lymph node	11±1.7	11±2.1	11±2.8	13±2.1	11±0.7	13±1.4	10±1.4	8±1.6
Mutant	Bone marrow	2±1.0	2±1.4	3±0.7	3±0.7	2±1.4	3±0.9	2±1.4	2±1.4
Wild type	Bone marrow	9±1.4	10±0.7	12±1.4	14±0.7	11±1.4	12±0.7	11±2.1	6±0.7

a) H-2 haplotype of each mouse strain.

b) Results were expressed as percentage of rosetting lymphoid cells ± standard deviation of three determinations.

In fact, BALB/c thymocytes rosetted well with the mutant erythrocytes even though these erythrocytes lack H-2L region controlled antigens. Presumably the receptors on BALB/c thymocytes primarily recognize H-2L antigens but weakly cross react with H-2K and H-2D antigens. This weak cross-reaction, combined with multi-point binding, would allow rosette formation to occur with the mutant erythrocytes.

Since the BALB/c-H-2^{dm2} strain represents an H-2L deletion mutant (McKenzie et al 1977, Hansen et al 1977), these data are consistent with the H-2L region playing an important role in autorosetting. On the other hand, it could be argued that a mutation in some other unrelated gene has modified the rosetting behaviour of the H-2^{dm2} mutant. To rule out this possibility another mutant mouse strain was examined, namely the B10.D2-H-2^{dm1} (M504) strain. This mutant has substantially modified H-2L and H-2D antigens (Morgan et al 1978). When this mutant was tested in the rosette-inhibition assay it was found that the H-2^{dm1} erythrocyte sonicate was unable to inhibit the autorosetting of H-2^{d}(BALB/c) thymocytes (Table 3.9). Conversely, H-2^{d} sonicates were unable to inhibit the autorosetting of H-2^{dm1} thymocytes. Similarly, it was found that H-2^{d} erythrocyte sonicates were unable to inhibit the autorosetting of H-2^{dm1} spleen, lymph node and bone marrow cells, whereas H-2^{dm1} sonicates could not inhibit the autorosettes formed by the lymphoid cells of the H-2^{d} haplotype (Table 3.10). Thus, these data are consistent with the notion that the lymphocyte autorosetting receptors primarily recognize self H-2L antigens on erythrocytes.

It should be noted from the experiment presented in Table 3.9 that the H-2^{dm1} mutant erythrocytes rosetted more effectively with thymocytes (either H-2^{d} or H-2^{dm1}) than H-2^{d} erythrocytes. The explanation of this effect is unclear. Presumably it is due to the different genetic background of BALB/c and B10.D2 mice, a point discussed in section 3.2.1.

Table 3.9

Specificity of Autorosetting-Inhibition of B10.D2 Mutant ($H\text{-}2^{dm1}$) [a] Thymocytes

Thymocyte	Erythrocyte	Erythrocyte Sonicate [b]		
		Nil	$H\text{-}2^{d}$	$H\text{-}2^{dm1}$
$H\text{-}2^{d}$	$H\text{-}2^{d}$	39±3.1	[6±2.7] [c]	37±2.1
$H\text{-}2^{dm1}$	$H\text{-}2^{dm1}$	57±3.4	54±1	[31±3.1]
$H\text{-}2^{d}$	$H\text{-}2^{dm1}$	55±2.9	[21±3.5]	51±2.6
$H\text{-}2^{dm1}$	$H\text{-}2^{d}$	37±2.8	36±2.5	[5±1.7]

a) B10.D2-$H\text{-}2^{dm1}$ strain also designated as M504 strain (Table I). In Table $H\text{-}2^{d}$ refers to BALB/c strain, $H\text{-}2^{dm1}$ to B10.D2 mutant ($H\text{-}2^{dm1}$) strain.

b) Thymocytes preincubated for 60 min at $4^{\circ}C$ with 100% erythrocyte sonicates prior to rosetting with appropriate erythrocytes.

c) Results expressed as % rosetting thymocytes ± standard deviation of three determinations. Values which represent significant inhibition are enclosed.

Table 3.10

Inhibition of Autorosetting of Lymphoid Cells from BALB/c and B10.D2 Mutant ($\underline{H\text{-}2}^{dm1}$)

Mice

Mouse Strain Rosetted a)	Lymphoid Organ Rosetted	Erythrocyte Sonicate b)		
		Nil	$\underline{H\text{-}2}^{d}$	$\underline{H\text{-}2}^{dm1}$
$\underline{H\text{-}2}^{d}$	Spleen	14 ± 1.4	[2 ± 2.5] c)	13 ± 1.4
$\underline{H\text{-}2}^{dm1}$	"	23 ± 1.4	21 ± 0.7	[6 ± 0.7]
$\underline{H\text{-}2}^{d}$	Lymph Node	12 ± 0.7	[3 ± 1.4]	13 ± 0.7
$\underline{H\text{-}2}^{dm1}$	" "	20 ± 2.8	19 ± 1.4	[5 ± 2.1]
$\underline{H\text{-}2}^{d}$	Bone Marrow	10 ± 1.4	[2 ± 1.4]	9 ± 2.1
$\underline{H\text{-}2}^{dm1}$	" "	14 ± 2.1	15 ± 0.7	[5 ± 0.7]

a) $H\text{-}2^{d}$ refers to BALB/c mice and $\underline{H\text{-}2}^{dm1}$ to B10.D2-$\underline{H\text{-}2}^{dm1}$ mutant mice.

b) Lymphoid cells preincubated for 60 min at 4°C with 100% erythrocyte sonicates prior to autorosetting.

c) Results expressed as % rosetting lymphoid cells ± standard deviation of mean. Values which represent significant inhibition are enclosed.

3.2.6 Variability in the BALB/c-H-2^{dm2} Mutant Mice

The autorosetting studies described above with the BALB/c-H-2^{dm2} mutant mice were performed with a line of the mutant that was bred and maintained at the John Curtin School of Medical Research. In early 1981, this line died out and fresh breeding stock was obtained from Professor I. F. C. McKenzie and Dr. R. Melvold. Unlike the original stock the thymocytes of these mice autorosetted as well as wild-type BALB/c thymocytes (Dr. Rylatt, personal communication, Sept., 1981). Additional dm2 mice obtained from Dr. Klein and Dr. Melief also autorosetted normally, although there were occasional mice (2 out of 12) that showed slightly lower thymocyte autorosetting percentages than the wild-type strain (34% versus 44%) (Nov., 1981 - April 1982).

The new mutant mice were then tested for their ability to reject wild-type skin grafts and respond to BALB/c spleen cells in MLC (Dr. Blanden, personal communication, Dec., 1981). It was found that variable MLC responses occurred, and out of ten mice skin grafted only two rejected the graft in 12 days, the remainder rejecting their grafts after 20-30 days. This is unlike the original dm2 mutant that consistently rejected wild-type grafts in 10-12 days, and which was origianlly defined by its ability to reject grafts in this manner (Melvold and Kohn 1976). Thus, the dm2 mutant appears to be an unstable deletion mutant of H-2L.

Support for mutant variablity was the observation that the original strain in Canberra bred poorly and was usually runted when compared with wild-type BALB/c. Furthermore, the mutant maintained this phenotype over a four year period. By contrast, the new dm2 mutants bred well and were never runted.

Attempts are being made to confirm mutant variability by examining variation in H-2L antigen expression in individual mice and

re-isolating the dm2 mutants bearing the phenotypes: runted, poor-breeding and non-autorosetting.

3.2.7 Genetic Mapping of Rosetting with Allogeneic and Xenogeneic Erythrocytes

It was important to determine whether the same thymocyte receptor also recognized allogeneic and xenogeneic erythrocytes. In order to answer this question, the genetics of rosette-inhibition when either allogeneic or xenogeneic (rat) erythrocytes were used as rosetting targets was examined. When CBA/H (H-2^k) thymocytes were reacted with allogeneic C57BL/6 (H-2^b) erythrocytes this rosetting showed the same genetics of inhibition as when autologous (CBA/H) erythrocytes were used (compared Figs. 3.2 and 3.5). Thus, CBA/H (H-2^k),BALB/c (H-2^d) , and A.TH erythrocyte sonicates were inhibitory whereas C57BL/6 (H-2^b), and C55 (H-2^{dm2}) sonicates did not inhibit (Fig. 3.5). In a similar manner, when CBA/H (H-2^k) thymocytes were rosetted with xenogeneic Lewis rat red cells the rosette inhibition was identical to that observed with autorosetting. Again, CBA/H, BALB/c, and A.TH sonicates lacked inhibitory activity (Fig. 3.5).

Collectively, these results indicate that the same thymocyte receptor (probably H-2L region controlled) interacts with syngeneic, allogeneic and xenogeneic (rat) erythrocytes.

3.2.8 Blocking of Autorosette-Inhibition with Anti-H-2 Sera

If thymocytes and spleen cells primarily recognize H-2L antigens on erythrocytes then erythrocyte sonicates should lose their ability to inhibit autorosetting if they are coated with anti-H-2L antibodies. To test this prediction BALB/c erythrocytes were preincubated with anti-H-$2L^d$ antibodies prior to sonication and used as inhibitors of autorosetting by BALB/c thymocytes and spleen cells.

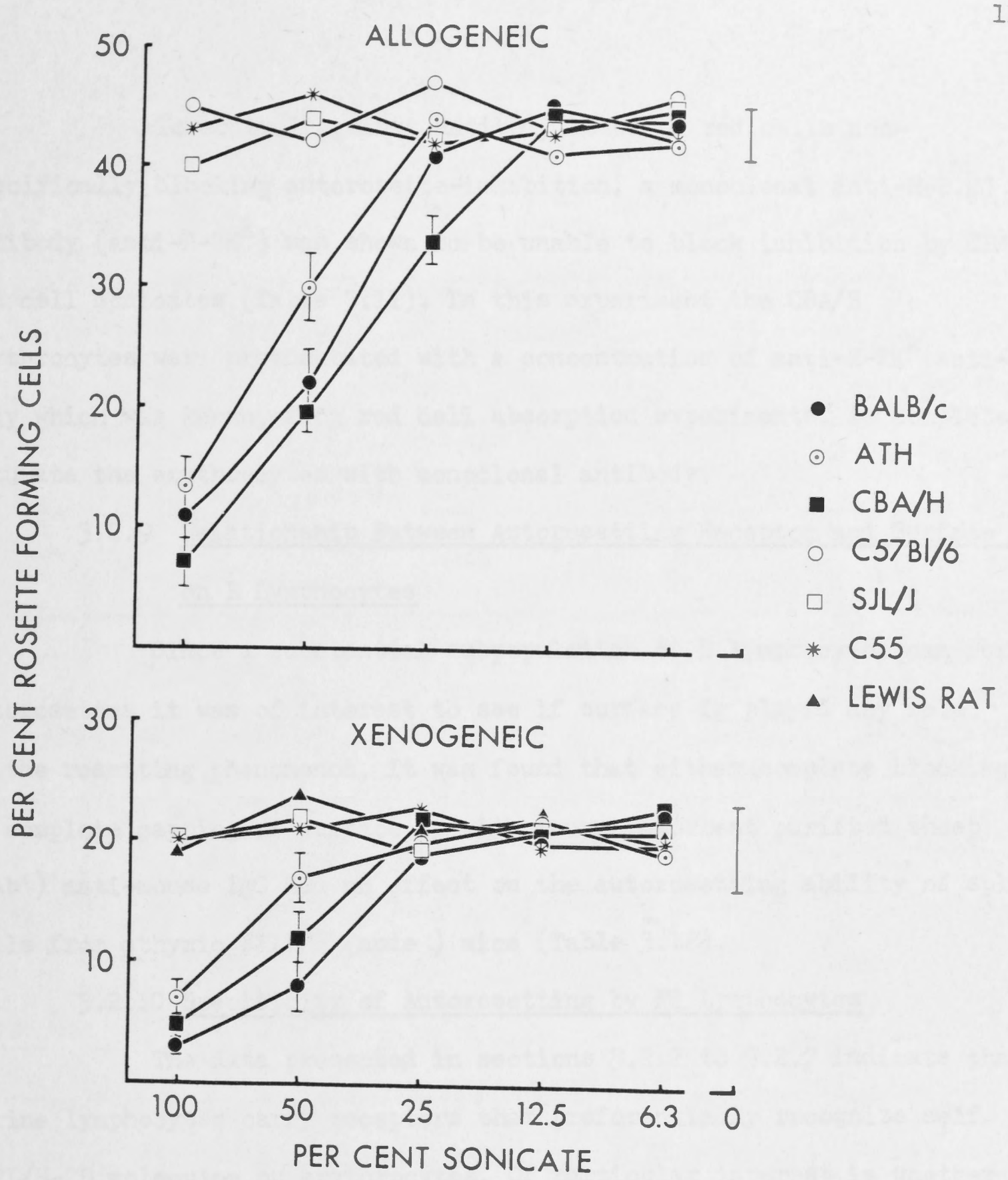

Fig. 3.5 Ability of erythrocyte sonicates from different mouse strains to inhibit the rosetting of CBA/H thymocytes with allogeneic (C57BL/6) and xenogeneic (Lewis rat) erythrocytes. The erythrocyte sonicates used as inhibitors are listed on the right hand side of the figure. Vertical bars represent standard deviations of means. The rosetting of control thymocytes, which were not preincubated with sonicates, is indicated in the right hand margin of each graph.

To control against antibody bound to red cells non-specifically blocking autorosette-inhibition, a monoclonal anti-H-2.25 antibody (anti-H-2K^k) was shown to be unable to block inhibition by CBA/H red cell sonicates (Table 3.11). In this experiment the CBA/H erythrocytes were preincubated with a concentration of anti-H-2K^k antibody which was known, from red cell absorption experiments, to completely saturate the erythrocytes with monoclonal antibody.

3.2.9 Relationship Between Autorosetting Receptor and Surface Ig on B Lymphocytes

Since a substantial subpopulation of B lymphocytes can form autorosettes it was of interest to see if surface Ig played any role in the rosetting phenomenon. It was found that either complete blocking or complete capping of surface Ig with immunoabsorbent purified sheep (Fab') anti-mouse IgG had no effect on the autorosetting ability of spleen cells from athymic BALB/c (nude) mice (Table 3.12).

3.2.10 Specificity of Autorosetting by F1 Lymphocytes

The data presented in sections 3.2.2 to 3.2.7 indicate that murine lymphocytes carry receptors that preferentially recognize self H-2L/H-2D molecules on erythrocytes. Of particular interest is whether in F1 hybrid mice, both parental receptors are expressed on all lymphocytes or exhibit allelic exclusion. The next series of experiments used inhibition of autorosetting by erythrocyte sonicates to examine this question.

Table 3.13 summaries the ability of different erythrocyte sonicates to inhibit the autorosetting of thymocytes from three F1 hybrid strains of mice. Fig. 3.6 depicts the complete inhibition data obtained with (CBA/H x C57BL/6)F1 thymocytes. It can be seen that autorosetting by (BALB/c x SJL/J)F1, (BALB/c x DBA/1)F1 and (CBA/H x C57BL/6)F1 thymocytes was strongly inhibited by the corresponding F1

Table 3.11

Blocking of Autorosette-Inhibition by Anti-H-2 Antibodies [a]

Autorosetting Cells	Antiserum Treatment of Sonicate [b]	Erythrocyte Sonicate [c]		
		None	BALB/c	CBA/H
Thymocytes	Nil	46 ± 4.0	9 ± 3.0	6 ± 2.1
Thymocytes	Anti-H-2L^{d}	-	43 ± 2.1	20 ± 2.5
Thymocytes	Anti-H-2K^{k}	-	8.5 ± 1.8	8 ± 2.3
Spleen Cells	Nil	16.5 ± 0.7	4.5 ± 0.7	4.0 ± 1.4
Spleen Cells	Anti-H-2L^{d}	-	15.0 ± 1.4	14.5 ± 0.7
Spleen Cells	Anti-H-2K^{k}	-	7.0 ± 1.4	6.5 ± 0.7

a) Inhibition of autorosetting between BALB/c thymocytes or spleen cells and erythrocytes.

b) Erythrocytes preincubated with antisera prior to sonication and used as inhibitors. Anti-H-2L^{d} = (BALB/c-H-2^{dm2} x A.SW)F1 anti-BALB/c serum. Anti-H-2K^{k} = monoclonal H-2.25 antibody (H100-27.R9).

c) Thymocytes or spleen cells preincubated for 60 min at 4°C with 100% erythrocyte sonicates prior to rosetting. Results expressed as % rosetting lymphocytes ± standard deviation of three determinations.

Table 3.12

Effect of Either Blocking or Capping Surface Immunoglobulin (Ig) on Autorosetting Potential [a] of B lymphocytes

Treatment [b]	% Autorosetting Spleen Cells
Nil	18.5 ± 2.1 [c]
Surface Ig Blocked	19.0 ± 1.4
Surface Ig Capped	20.5 ± 3.5

a) Spleen cells from BALB/c nude mice were autorosetted.

b) Spleen cells were incubated with immunoabsorbent purified sheep $(Fab')_2$ anti-mouse IgG either on ice (for blocking) or at 37°C (for capping).

c) Mean autorosetting percentage ± standard deviation of mean.

Table 3.13

Ability of Different Erythrocyte Sonicates to Inhibit Autorosetting of F1 Thymocytes

Thymocyte Autorosetted	Erythrocyte Sonicate [a]								
	Nil	(BALB/c X SJL/J)F1	(BALB/c X DBA/1)F1	(CBA/H X C57BL/6)F1	BALB/c (d) [c]	SJL/J (s)	DBA/1 (q)	CBA/H (k)	C57BL/6 (b)
(BALB/c X SJL/J)F1	42 ±3.2	[7 ±3.5] [b]	ND	ND	[6 ±4.2]	[28 ±3.5]	43 ±3.0	[7 ±3.6]	41 ±3.6
(BALB/c X DBA/1)F1	53 ±3.7	ND	[16 ±2.9]	ND	[12 ±3.7]	53 ±3.1	[13 ±3.4]	[6 ±3.7]	53 ±2.6
(CBA/H X C57BL/6)F1	47 ±2.8	ND	ND	[8 ±3.8]	[7 ±4.4]	44 ±3.9	45 ±3.2	[4 ±3.7]	[21 ±3.6]

a) Thymocytes preincubated for 60 min at 4°C. with 100% erythrocyte sonicate prior to rosetting with autologous erythrocytes.

b) Results expressed as % rosetting thymocytes ± standard deviation of three determinations. Values which represent significant inhibition are enclosed.

c) H-2 haplotype of each mouse strain.

ND Not determined.

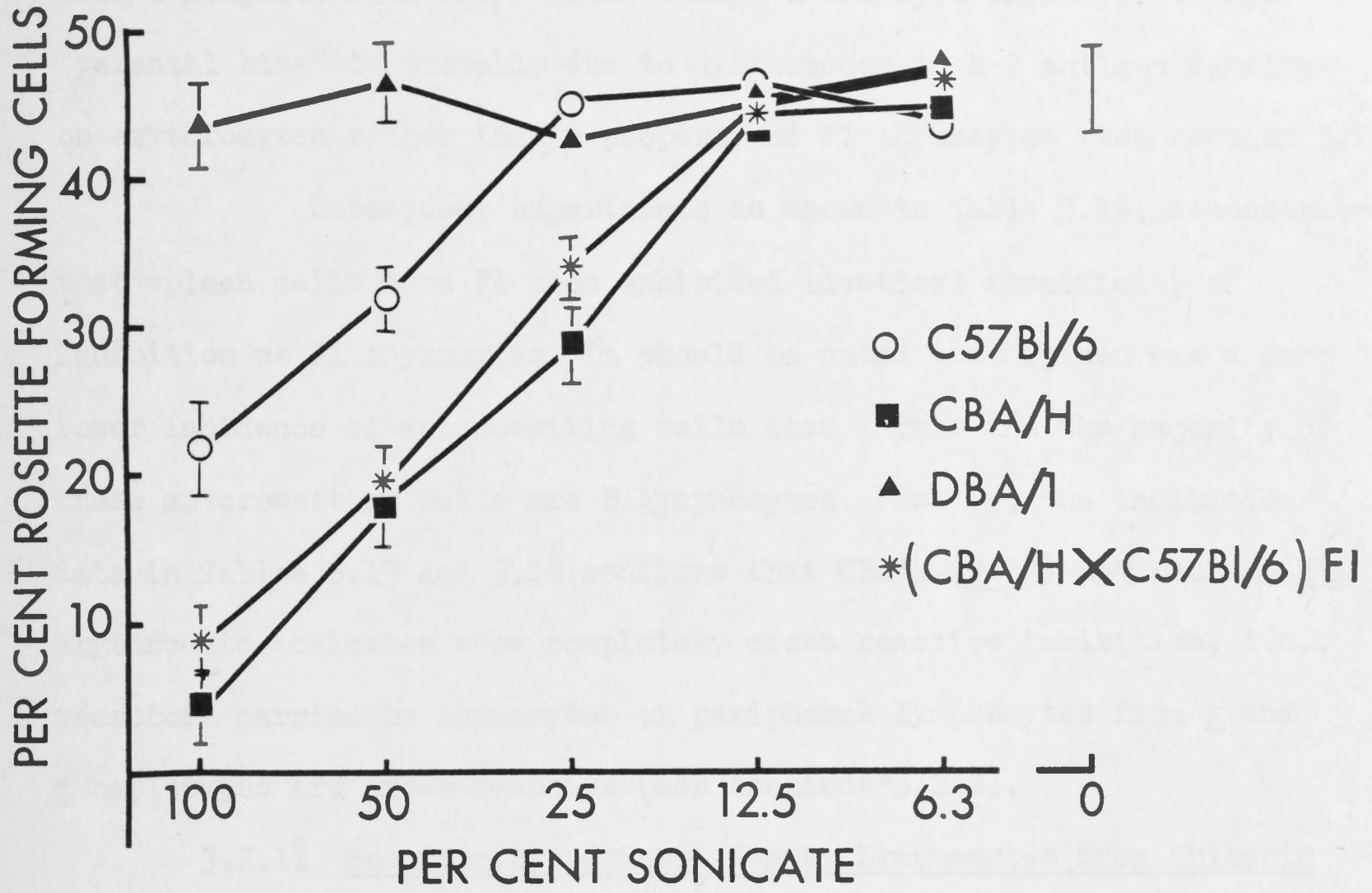

Fig. 3.6 Ability of erythrocyte sonicates from different mouse strains to inhibit the rosetting of (CBA/H X C57BL/6)F1 thymocytes with (CBA/H X C57BL/6)F1 erythrocytes. The erythrocyte sonicates used as inhibitors are listed on the figure. Thymocytes were preincubated with different concentrations of sonicates prior to rosetting. Vertical bars represent standard deviations of means. The rosetting of control thymocytes, which were not preincubated with sonicates, is indicated in the right hand margin of the figure.

erythrocyte sonicates. Furthermore, sonicates of the parental strain erythrocytes were effective inhibitors of autorosetting, although in some cases sonicates of one parent inhibited better than the other, e. g., BALB/c compared with SJL/J with (BALB/c x SJL/J)F1 thymocytes. This "parental bias" is probably due to differences in H-2 antigen density on erythrocytes rather than a property of F1 thymocytes (see section 3.2.3).

Subsequent experiments as shown in Table 3.14, demonstrated that spleen cells from F1 mice exhibited identical specificity of inhibition as F1 thymocytes. It should be noted that spleen has a much lower incidence of autorosetting cells than thymus and the majority of these autorosetting cells are B lymphocytes. Finally, the inhibition data in Tables 3.13 and 3.14 confirms that CBA/H ($\underline{H\text{-}2}^{k}$) and BALB/c ($\underline{H\text{-}2}^{d}$) erythrocyte sonicates show completely cross reactive inhibition, i.e., receptors carried by thymocytes or peripheral lymphocytes from *k* and *d* haplotypes are cross reactive (see sections 3.2.2).

3.2.11 Specificity of Rosetting by Lymphocytes from Chimeric Mice

In order to establish whether the thymic epithelium dictates the specificity of the H-2L region restricted receptors on thymocytes and spleen cells, three types of chimeric mice were examined, namely: F1 into parent; parent into F1 and completely allogeneic chimeras. The specificity of the chimeric thymocytes, as measured by inhibition of rosetting by erythrocyte sonicates, is presented in Table 3.15. In all three types of chimeras the thymocytes gave the same pattern of inhibition as the donor cells rather than as the X-irradiated recipient. Thus, with the completely allogeneic chimera, C57BL/6 → CBA/H, inhibition of rosetting was achieved only with sonicates derived from C57BL/6 or (C57BL/6 x CBA/H)F1 mice. Similarly, with the (C57BL/6 x CBA/H)F1 → CBA/H chimera both parental sonicates

Table 3.14

Ability of Different Erythrocyte Sonicates to Inhibit Autorosetting of F1 Spleen Cells

Spleen Cell Autorosetted	Erythrocyte Sonicate[a]								
	Nil	(BALB/c X SJL/J)F1	(BALB/c X DBA/1)F1	(CBA/H X C57BL/6)F1	BALB/c (d)[c]	SJL/J (s)	DBA/1 (q)	CBA/H (k)	C57BL/6 (b)
(BALB/c X SJL/J)F1	11±2.2	1±1.7[b]	ND	ND	1±1.5	3±2.2	8±2.7	2±1.2	9±1.9
(BALB/c X DBA/1)F1	10±2.4	ND	1±1.3	ND	2±1.6	9±2.1	2±2.1	1±1.4	11±2.3
(CBA/H X C57BL/6)F1	7±2.1	ND	ND	1±1.4	1±1.7	8±1.5	7±2.4	2±1.3	3±1.6

a) Spleen cells preincubated for 60 min at 4°C. with 100% erythrocyte sonicates prior to rosetting with the erythrocytes.

Footnotes as in Table 3.13

Table 3.15

Ability of Erythrocyte Sonicates to Inhibit Rosetting of Thymocytes from Chimeric and Normal Mice

Thymocyte Rosetted	Erythrocyte	Erythrocyte Sonicate [a]				
		Nil	CBA/H (k) [c]	C57BL/6 (b)	DBA/1 (q)	(C57BL/6 X CBA/H)F1
C57BL/6 → CBA/H	CBA/H	34±2.7	27±1.7	[17±2.8] [b]	29±2.9	[12±2.8]
C57BL/6	CBA/H	33±2.8	30±2.4	[14±2.5]	31±2.5	[15±3.1]
(C57BL/6 X CBA/H)→ CBA/H	CBA/H	33±2.9	[10±2.7]	[18±3.4]	31±3.0	[9±3.2]
(C57BL/6 X CBA/H)F1	CBA/H	32±3.5	[9±3.2]	[16±3.6]	29±3.3	[14±3.4]
CBA/H → (C57BL/6 X CBA/H)F1	(C57BL/6 X CBA/H)F1	46±3.4	[19±2.5]	47±3.6	45±3.8	[17±4.2]
CBA/H	(C57BL/6 X CBA/H)F1	45±4.2	[8±4.3]	38±3.8	44±2.9	[11±2.8]

a) Thymocytes preincubated for 60 min at 4°C. with 100% erythrocyte sonicates prior to rosetting with the erythrocytes.

Footnotes as in Table 3.13

were inhibitory whereas with the CBA/H → (C57BL/6 x CBA/H)F1 chimeras only CBA/H derived erythrocyte sonicates inhibited rosetting. It should be emphasized that the same specificity of sonicate inhibition was obtained whether chimeric thymocytes were rosetted with CBA/H, C57BL/6 or (C57BL/6 x CBA/H)F1 erythrocytes (data not shown). This result is consistent with the earlier finding that, although thymocytes can rosette with allogeneic erythrocytes, inhibition of rosetting was only achieved with syngeneic erythrocyte sonicates (see section 3.2.6).

In similar experiments, as shown in Table 3.16, it was found that spleen cells from these chimeras showed the same specificity of inhibition as the thymocytes.

3.3 Discussion

3.3.1 Nature of Autorosetting Cells

Substantial subpopulations of murine thymocytes, spleen, lymph node and bone marrow cells can bind autologous, syngeneic or allogeneic red cells (Micklem and Asfi 1971, Charriere and Bach 1975, Braganza *et al* 1975, Kolb 1977, 1978, Nabarra and Charriere 1979). However, the proportion of cells that autorosette in different lymphoid organs varies between laboratories due to important assay variables that have been described in detail in chapters 1 (section 4.1) and 2 (section 2.10.1).

Using a standardised rosetting assay the frequency of autorosetting cells in different lymphoid organs was thymus > spleen > lymph node > bone marrow (Table 3.1). A comparatively high proportion of thymocytes formed rosettes (20 -50%) and up to 30% of spleen cells autorosetted. However, autorosetting efficiency did vary between mouse strains, an effect that appeared to be controlled by background (non-*H-2*) genes and resided in the rosetting erythrocyte.

Table 3.16

Ability of Erythrocyte Sonicates to Inhibit Rosetting of Spleen Cells from Chimeric and Normal Mice

Spleen Cell Rosetted	Erythrocyte	Erythrocyte Sonicate[a]				
		Nil	CBA/H (k)[c]	C57BL/6 (b)	DBA/1 (q)	(C57BL/6 X CBA/H)F1
C57BL/6 → CBA/H	CBA/H	7±2.2	7±1.4	1±1.2[b]	8±2.0	2±1.4
C57BL/6	CBA/H	9±2.4	8±1.8	3±1.3	7±1.7	1±1.1
(C57BL/6 X CBA/H) → CBA/H	CBA/H	7±1.8	1±1.0	3±1.4	8±1.7	1±1.3
(C57BL/6 X CBA/H)F1	CBA/H	8±1.4	1±1.6	4±1.5	7±2.1	1±0.9
CBA/H → (C57BL/6 X CBA/H)F1	(C57BL/6 X CBA/H)F1	7±2.1	1±0.9	8±1.6	7±2.2	1±1.2
CBA/H	(C57BL/6 X CBA/H)F1	8±1.7	1±0.7	7±1.9	7±2.4	2±1.1

a) Spleen cells preincubated for 60 min at 4°C. with 100% erythrocyte sonicates prior to rosetting with the erythrocytes.

Footnotes as in Table 3.13

Although thymocyte autorosetting indicated that immature T lymphocytes carry autorosetting receptors, cell separation experiments demonstrated that in fact, the majority (80%) of autorosetting cells in spleen are B lymphocytes, whereas approx. 15% of T cells and few (< 1%) null cells can autorosette. However, Ig capping and capping studies clearly demonstrated that autorosetting by B lymphocytes is mediated by recognition structures distinct from surface Ig (Table 3.12). Similar results have been reported by others (Primi et al 1979, Kolb 1978).

3.3.2 Specificity of Autorosetting Receptors

In this chapter the specificity of the autorosetting phenomenon was analysed by an inhibition assay that entailed incubating lymphoid cells with different erythrocyte sonicates prior to rosetting the lymphocytes with autologous erythrocytes. With this assay it was demonstrated that autorosetting is mediated by a receptor on thymocytes, as well as on peripheral T and B lymphocytes which preferentially recognize self H-2L/H-2D region controlled molecules on the red cell surface.

Data in favour of this conclusion can be summarised as follows. First, inhibition of thymocyte and spleen cell autorosetting in seven different mouse strains suggested H-2 linked rather than background genes controlled the reaction (Tables 3.3 - 3.5). Second, using erythrocyte sonicates from a range of recombinant mouse strains it was possible to map the inhibition to the H-2L/H-2D region of the H-2 complex (Fig. 3.2 - 3.4). Third, the inability of the BALB/c-H-2^{dm2} mutant, an H-2L deletion mutant (McKenzie et al 1977, Démant and Néauport-Sautès 1978), to inhibit the autorosetting of wild type (BALB/c) thymocytes and spleen cells directly mapped the specificity of autorosetting to the H-2L region (Fig. 3.2 & 3.4). Similarly, the B10.D2-H-2^{dm1} mutant (M504), which has substantially modified H-2L and H-2D antigens

(Morgan et al 1978), was unable to inhibit wild type H-2^d (BALB/c) rosetting and expressed a different specificity receptor on its thymocytes (Tables 3.9 and 3.10). Fourth, anti-H-2L sera were able to specifically block the inhibition of BALB/c autorosetting by sonicates of BALB/c erythrocytes (Table 3.11).

The specificity of autorosetting of five different haplotypes, namely b, d, k, q and s, were examined in this study. The inhibition data suggested complete cross reaction between the lymphocyte receptors carried by the k and d haplotypes. This result implies that autorosetting lymphocytes cannot distinguish between the H-2L molecules carried by the d and k haplotypes even though there are serologically detectable differences between these H-2L molecules (McKenzie et al 1977, Demant and Neauport Sautes 1978, Hansen and Sachs 1978).

Of course, due to the lack of suitable recombinant mouse strains, mutant mice and alloantisera it was not possible to directly map the specificity of the autorosetting receptors to the H-2L region in all five haplotypes. However, data for the d and k haplotypes implicated the H-2L region in autorosetting. With the s haplotype the receptor was mapped to the H-2L/H-2D region, whereas with the b and q haplotypes autorosetting was mapped to the D end of the H-2 complex (i.e., to the right of the S region).

Rosette-inhibition results presented in this chapter also suggest that the receptors that recognize self H-2L/H-2D structures on red cells also mediate the rosetting of thymocytes with allogeneic and xenogeneic (rat) erythrocytes (Fig. 3.5). This conclusion is based on the observation that when CBA/H (H-2^k) thymocytes were reacted with allogeneic (C57BL/6, H-2^b) or xenogeneic (Lewis rat) erythrocytes this rosetting showed the same genetics of inhibition as when autologous (CBA/H) red cells were used (Fig. 3.5). The inability of thymocytes,

spleen, lymph node and bone marrow cells from the H-2L deletion mutant ($H\text{-}2^{dm2}$) to rosette with any red cells (Tables 3.7 and 3.8) is consistent with this notion.

Collectively these results suggest that rosetting of murine lymphocytes with syngeneic, allogeneic and xenogeneic (rat) erythrocytes is mediated by an H-2L/H-2D region controlled receptor that has highest affinity for syngeneic H-2L/H-2D antigens on erythrocytes but which can cross react with other H-2 antigens on the red cell surface. Inhbition studies with erythrocyte sonicates revealed this "self H-2L/H-2D preference" probably for two reasons: (i) binding of intact red cells represents multi-point binding whereas the membrane vesicles generated by sonication would probably not bind in such a multi-point fashion and (ii) unbound sonicate was washed away from lymphocyte whereas it was impractical to remove unbound red cells in rosetting assays. The ability of sonicate-inhibition studies to reveal "self-preference" is probably most clearly demonstrated by the rosetting behaviour of the B10.D2-$H\text{-}2^{dm1}$(M504) mutant erythrocytes. Sonicates of these red cells are unable to inhibit wild-type ($H\text{-}2^{d}$) autorosetting (Fig. 3.2 and 3.4) whereas when intact these mutant red cells rosette normally with the autologous and syngeneic (BALB/c) thymocytes (Tables3.7&3.8)

At this point it should be noted that two recent reports have appeared describing attempts to map the genes responsible for autorosetting. In the first report, Primi et al concluded that rosetting was under H-2 control but from their data, suggested that this self-recognition phenomenon was associated with a new locus mapping between H-2G and H-2L/H-2D. Data presented in this chapter are not completely compatible with this interpretation, although there are substantial differences between the assay systems used in the two laboratories. First, Primi et al used cultured spleen cells whereas

freshly prepared thymocytes and spleen cells were used in all the experiments described in this chapter. Even so, uncultured spleen cells carry H-2L/H-2D region controlled receptors. Second, in this chapter the autorosetting receptors have been genetically mapped using mutant mice and autorosette-inhibition assays, whereas Primi et al based their genetic mapping on the observation that cultured spleen cells could only rosette with erythrocytes from certain mouse strains. This is a surprising result as uncultured thymocytes and spleen cells rosette equally effectively with erythrocytes from autologous, syngeneic, or allogeneic strains (Micklem and Asfi 1971, Sandilands et al 1974, Charriere and Bach 1975, Braganza et al 1975, Kolb 1977). At present, the only rational explanation of these differences is that cultured spleen cells recognize different structures on erythrocytes than their uncultured counterparts.

In the second study, Charriere et al (1980) employed two approaches to determine the role of the H-2 complex in autorosetting. First, like Primi et al (1979), they observed that lymphocytes preferentially bound H-2 compatible erythrocytes.The second approach was to measure the ability of H-2 compatible or incompatible erythrocyte ghosts to inhibit autorosetting. Based on the data of one H-2 recombinant mouse strain B10.A (K^k, D^d), they concluded that both K and D regions were involved in autorosetting. However, this study only examined one recombinant between k and d haplotypes, and the data in this chapter clearly demonstrated that the autorosetting receptors of k and d haplotypes are completely cross-reactive, i.e., genetic mapping studies cannot be attempted between these haplotypes.

3.3.3 Expression of Autorosetting Receptors on F1 Lymphocytes

Analysis of the specificity of the autorosetting receptors in F1 mice using the sonicate-inhibition approach, revealed that both the thymocytes and peripheral lymphocytes in these animals express receptors against both parental haplotypes. In fact, the sonicate inhibition data in Tables 3.13 & 3.14 suggest that the majority of thymocytes and spleen cells simultaneously express receptors against both parents. This lack of allelic exclusion is clearly evident with (BALB/c x DBA/1)F1 lymphocytes where erythrocyte sonicates from either BALB/c or DBA/1 mice reduced rosetting by 75 -80%. Similarly, with (CBA/H x C57BL/6)F1 lymphocytes CBA/H and C57BL/6 sonicates inhibited rosetting by 70 - 90% and 55 - 60% respectively. Presumably once lymphocyte receptors against one parental haplotype bind sonicated erythrocytes the receptors directed against the other parent are sterically masked. This notion is supported by the observation that erythrocyte sonicates are membrane vesicle preparations, and not solubilised membrane components, i. e., the vesicles that inhibit autorosetting are sedimented by centrifugation at between 5000 g and 20,000 g. Of course, this study does not eliminate the possibility that small subpopulations of F1 thymocytes exist which carry receptors directed against only one of the parents.Furthermore, whether F1 lymphocytes exhibit allelic exclusion of their receptors when they become immunocompetent or after antigenic stimulation remains to be determined.

3.3.4 General Implications of MHC-Controlled Autorosetting Receptors

Although the genetic and functional implications of the data presented in this chapter will be discussed in chapter 7 some of the more important points are worth considering here.

First, the data suggests that the H-2L/H-2D region controls both the autorosetting receptors on thymocytes and the acceptor sites on erythrocytes. The results with the H-2L/H-2D region mutant mice, which simultaneously express altered autorosetting receptors and acceptors, clearly supports this concept. There appear to be two possible explanations for these findings: (i) the H-2L/H-2D gene product stimulates, possibly via somatic mutation, the generation of a receptor which recognizes self H-2L/H-2D. This possibilty appears unlikely, however, as analysis of lymphocytes from allogeneic and semi-allogeneic chimeras (Tables 3.15 & 3.16) clearly demonstrated that radio-resistant elements of the irradiated recipient did not modify the haplotype specificity of the autorosetting receptors on donor derived lymphocytes. (ii) more likely interpretation is that the H-2L/H-2D region in some way directly codes for the autorosetting receptors and acceptors. How one genetic region can simultaneously code for these interacting structures will be considered in detail in chapter 7.

Second, a surprising aspect of this study is the dominance of H-2L/H-2D region controlled receptors on autorosetting lymphocytes. From the studies with cytotoxic T cells against foreign antigens, one would have expected to also detect autorosetting receptors on lymphocytes against self H-2K and self H-2D antigens (Doherty et al 1976, Biddison et al 1978). This observation strongly suggests that the autorosetting receptors are not involved in H-2-restricted killing of targets by Tc cells. Furthermore, unlike Tc cells, the haplotype specificity of the autorosetting cells was not altered in allogeneic and semi-allogeneic chimeras (Tables 3.15 & 3.16).

The functional significance of the autorosetting receptors on T and B lymphocytes represents a fascinating question which

at present, cannot be answered. Undoubtedly these recognition structures appear on immature T and B lymphocytes as substantial subpopulations of thymocytes and bone marrow cells carry the receptors. Whether immunologically competent lymphocytes also express the receptors needs to be established. It should be noted, however, that any function of these autorosetting receptors must be tightly regulated *in vivo* by blocking factors in serum (Kolb 1977), a point that will be discussed in more detail in chapter 6. On the other hand, the autorosetting receptors may be involved in a range of 'odd' immunological phenomenon that have been reported in the past. For example, the species-specific attachment of thymocytes to macrophages *in vitro* may be mediated by these receptors (Siegel 1970, Lipsky and Rosenthal 1973). At a more functional level, it is conceivable that the phenomenon of "hybrid resistance", where irradiated F1 hybrid recipients reject parental bone marrow (Cudkowicz and Bennett 1971), involves the autorosetting receptors. Furthermore, the self-reactive cytotoxic T cells which frequently appear following *in vitro* culture (Cohen and Wekerle 1973) or after viral infections (Pfizenmaier *et al* 1975, Komatsu 1978) may recognize target cells via receptors for self H-2L/H-2D. Finally, although only 1% null cells in spleen can autorosette, the general properties of natural killer cells (Lotzova and Mccredie 1978) suggest that they may interact with target cells via recognition structures which primarily recognize self H-2L/H-2D antigens.

Finally, it should be noted that previous studies have demonstrated autorosetting lymphocytes in a range of other species, such as rats, rabbits, pigs and humans (Sandilands *et al* 1974, Braganza *et al* 1975, Kolb 1977, Baxley *et al* 1975). It seems likely that MHC-controlled receptors may also mediate autorosetting in these species. Furthermore,

in some mammalian species the binding of xenogeneic erythrocytes by lymphoid cells (Braganza et al 1975, Kolb 1977, Jondal et al 1972, Stadecker et al 1973) may be mediated by MHC-controlled receptors.

3.4 Summary

Subpopulations of murine spleen, lymph node and bone marrow cells can bind autologous erythrocytes. The specificity of this interaction was investigated by measuring the ability of different erythrocyte sonicates to inhibit rosette formation. Using erythrocyte sonicates from recombinant mouse strains it was demonstrated that rosetting with syngeneic red cells was mediated by receptors on thymocytes and peripheral T and B lymphocytes which preferentially recognize self H-2L/H-2D region controlled molecules on the red cell surface. The specificity of autorosetting was directly mapped to the H-2L region by the inability of erythrocyte sonicates from the BALB/c-H-2^{dm2} mutant, an H-2L deletion mutant, to inhibit the rosetting of wild type (BALB/c) lymphocytes. The B10.D2-H-2^{dm1} mutant, which has substantially modified H-2L and H-2D antigens, supported this conclusion. Furthermore, anti-H-2L sera were able to specifically block the inhibition of rosetting by erythrocyte sonicates.

The above procedures clearly implicated the H-2L region in the lymphocyte rosetting of d and k haplotypes. With the s haplotype the rosetting was mapped to the H-2L/H-2D region, whereas with the b and q haplotypes rosetting was only mapped to the D end of the H-2 complex. This study also suggested complete cross reaction between the autorosetting receptors carried by the k and d haplotypes, whereas the receptors of b, q, and s haplotypes were haplotype specific. In addition, the inhibition assay indicated that the rosetting of thymocytes and extra-thymic

lymphocytes with allogeneic and xenogeneic (rat) erythrocytes was mediated by a receptor primarily directed against self H-2L/H-2D molecules.

Studies with H-2L/H-2D mutant mice also demonstrated that autorosetting is under H-2L/H-2D region control.

The specificity of autorosetting also was examined in F1 hybrid and chimeric mice by inhibiting autorosetting with different erythrocyte sonicates. It was found that the majority of F1 lymphocytes simultaneously express receptors against both parental haplotypes. Furthermore, analysis of lymphocytes from allogeneic and semi-allogeneic chimeras clearly demonstrated that radioresistant elements in the irradiated recipient did not modify the haplotype specificity of the receptor on donor derived lymphocytes.

Finally, the expression of autorosetting receptors on splenic T and B cells was examined. It was found that the majority of autorosetting lymphocytes in spleen were B cells, fewer T cells (15%) and virtually no (< 1%) null cells autorosette. Futhermore, the autorosetting receptors on B lymphocytes were distinct from surface Ig and could develop in athymic (nude) mice.

Chapter 4

Autorosetting Receptors on Thymocytes Recognize Carbohydrate Structures on Erythrocytes

Table of Contents

4.1 Introduction

In the preceding chapter, the autorosetting phenomenon was demonstrated to be mediated by H-2-controlled receptors on thymocytes and extra-thymic lymphocytes that primarily recognize self H-2L/H-2D molecules on erythrocytes. Obviously this autorosetting system represents an ideal opportunity to chemically characterize the receptor and acceptor molecules involved in an H-2-controlled interaction. This chapter describes preliminary attempts to characterize these molecules. On the basis of sugar inhibition studies and the sensitivity of the receptors and acceptors to protease and glycosidase treatments it appears that a protein receptor on thymocytes recognize a carbohydrate structure on erythrocytes.

4.2 Results

4.2.1. Inhibition of Thymocyte Rosetting with Sugars

The thirty monosaccharides and oligosaccharides listed in chapter 2, Table 2.2 were tested for their ability to inhibit the rosetting of different strains of thymocytes with CBA/H erythrocytes. The sugars tested for inhibitory activity represent the same haptens used previously to partially characterize Ia antigens (McKenzie et al 1977). It should be noted that it has previously been shown that erythrocytes from different mouse strains vary in their rosetting behaviour, a phenomenon dependent upon the genetic background rather than the H-2 haplotype of the erythrocytes (chapter 3, section 3.2.1). Thus, in order to directly compare the sugar inhibition observed with different strains of thymocytes, all thymocytes were rosetted with CBA/H erythrocytes.

The eight sugars that showed inhibitory activity are listed in Table 4.1. It was found that the monosaccharides D-galactosamine, D-mannose and N-acetylneuraminic acid and the oligosaccharides raffinose, maltose and mannan inhibited the rosetting of all mouse strains tested. Mannan, a

Table 4.1

Ability of Different Sugars to Inhibit the Rosetting of Different Thymocytes with CBA/H Erythrocytes

Sugar Inhibitor	Strain of Origin of Thymocytes [a]										
	CBA/H (k) [b]	B10.BR (k)	DBA/1 (q)	B10.G (q)	DBA/2 (d)	BALB/c (d)	B10.D2 (d)	C57BL/6 (b)	C57BL/10 (b)	SJL/J (s)	B10.d2 mutant (dml)
Monosaccarides:											
D-glucose	-	-	-	-	-	-	-	-	-	-	-
D-galactose	-	-	-	-	-	-	-	-	-	44±6.5 [c]	32±5.4
D-galactosamine	66±6.6	73±7.1	63±6.9	72±7.4	64±6.9	70±6.9	71±5.7	61±6.7	70±7.1	72±6.5	67±5.4
D-mannose	74±4.4	77±4.6	79±5.1	68±4.3	76±3.6	71±5.5	67±4.8	74±3.9	75±4.1	70±6.2	66±4.6
D-mannosamine	-	-	52±6.2	60±7.5	-	-	-	62±6.0	56±6.1	-	-
N-acetyl-neuraminic acid	66±6.5	67±5.2	54±4.7	66±6.5	59±4.5	67±6.2	65±4.2	70±5.8	65±5.5	68±5.1	63±5.4
Oligosaccarides:											
raffinose	49±5.7	51±5.2	48±5.6	47±4.8	57±5.4	53±4.9	49±5.1	41±6.4	43±4.9	52±4.6	52±5.3
maltose	58±6.3	54±6.2	60±6.2	48±5.9	46±5.3	62±5.6	64±4.8	53±5.9	56±6.2	64±5.5	59±4.9
mannan	84±4.6	87±6.5	87±4.9	82±5.3	85±6.2	89±4.5	79±5.2	81±5.7	84±4.6	86±5.4	81±6.1

a) Thymocytes preincubated for 60 min at 4°C with sugar (10mg/ml) prior to rosetting with CBA/H erythrocytes.

b) *H-2* haplotype of mouse strains.

c) Results expressed as % inhibition of rosetting ± standard deviation of three determinations. A dash means no significant inhibition of rosetting.

polymer of mannose, was the most effective of these inhibitory sugars. On the other hand, at 10 mg/ml D-galactose and mannosamine exhibited strain specific inhibition. Thus, D-galactose only inhibited the rosetting of SJL/J ($\underline{H\text{-}2}^{s}$) and B10.D2 mutant ($\underline{H\text{-}2}^{dm1}$) thymocytes, whereas D-mannosamine inhibited the rosetting of thymocytes from both $\underline{H\text{-}2}^{q}$ (DBA/1 and B10.G) and $\underline{H\text{-}2}^{b}$ (C57BL/6 and C57BL/10) haplotypes.

The effect of a range of sugar concentrations on the rosetting of DBA/1 and CBA/H thymocytes is depicted in Fig. 4.1. It can be seen that D-galactosamine and mannan inhibited the rosetting of both strains. In contrast, D-mannosamine strongly inhibited the rosetting of DBA/1 thymocytes but, only at very high concentrations (i.e., 20 mg/ml), showed slight inhibition of CBA/H rosetting. The monosaccharides D-glucose and D-galactose were non-inhibitory at all concentrations used.

Although previous studies have shown that rosetting is mediated by H-2L/H-2D region controlled receptors on thymocytes (chapter 3), it was important to confirm that the differences in sugar inhibition were H-2 controlled. Certainly, the data in Table 4.1 suggested H-2 involvement as different B10 congenic strains of mice exhibited different patterns of sugar inhibition, i.e., mannosamine inhibited B10.G ($\underline{H\text{-}2}^{q}$) and B10 ($\underline{H\text{-}2}^{b}$) rosetting, but failed to block B10.BR ($\underline{H\text{-}2}^{k}$) and B10.D2 ($\underline{H\text{-}2}^{d}$) rosetting. Furthermore, comparable sugar inhibition was observed with thymocytes from mouse strains of the same H-2 haplotype but of different genetic background, e.g. DBA/1 and B10.G ($\underline{H\text{-}2}^{q}$). In addition, rosetting of B10.D2-$\underline{H\text{-}2}^{dm1}$ mutant thymocytes was inhibited by D-galactose, whereas the rosetting of wild-type B10.D2 thymocytes was not inhibited by this sugar. Since this mutant has substantially modified H-2L and H-2D antigens (Morgan et al 1978) this result suggests that the sugar inhibition is H-2L/H-2D region controlled.

Further genetic analysis of the inhibition of rosetting by

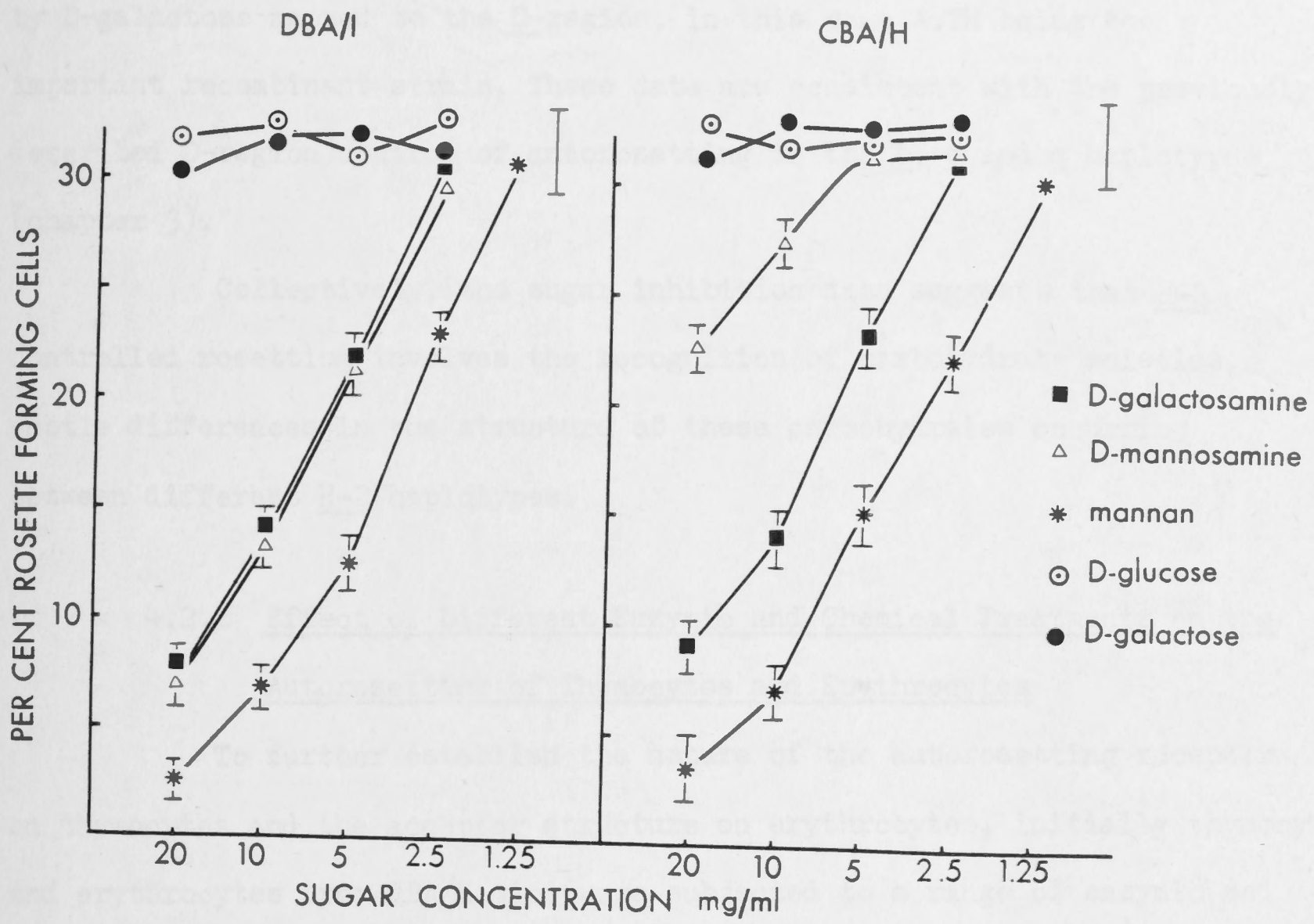

Fig. 4.1 Ability of different concentrations of sugars to inhibit the rosetting of either DBA/1 (left hand graph) or CBA/H (right hand graph) thymocytes with CBA/H erythrocytes. The sugars used as inhibitors are listed on the right hand side of the figure. Vertical bars represent standard deviations of means. The rosetting of thymocytes, in absence of inhibitors, is indicated in the right hand margin of each graph.

D-mannosamine is presented in Table 4.2. Inhibition of autorosetting by D-mannosamine mapped to the D-end of the MHC for both q and b haplotypes. The critical recombinant for the haplotype was B10.A(2R) and for the q haplotype, B10.T(6R). Similarly, the inhibition of s haplotype rosetting by D-galactose mapped to the D-region, in this case A.TH being the important recombinant strain. These data are consistent with the previously described D-region control of autorosetting in the b, q and s haplotypes (chapter 3).

Collectively, the sugar inhibition data suggests that H-2 controlled rosetting involves the recognition of carbohydrate moieties, subtle differences in the structure of these carbohydrates occurring between different H-2 haplotypes.

4.2.2 Effect of Different Enzymic and Chemical Treatments on the Autorosetting of Thymocytes and Erythrocytes

To further establish the nature of the autorosetting receptor on thymocytes and the acceptor structure on erythrocytes, initially thymocytes and erythrocytes from CBA/H mice were subjected to a range of enzymic and chemical treatments (Table 4.3). It was found that both the thymocyte receptor and the erythrocyte acceptor were almost completely abolished by the proteases bromelin, pronase and papain and partially destroyed by trypsin. In contrast, mixed glycosidases destroyed the erythrocyte acceptor but had no effect on the thymocyte receptor. A number of selective exo-glycosidases also partially destroyed the erythrocyte acceptor, namely β-galactosidase, neuraminidase and α-mannosidase, whereas the acceptor was resistant to α-galactosidase. None of these glycosidases affected the thymocyte receptor. Furthermore, the erythrocyte acceptor, but not the thymocyte receptor, was susceptible to oxidation by low concentration (10^{-4}M) of periodate. Thus, the data in Table 4.3 suggested that a protein sensitive receptor on thymocytes recognized a protein-bound carbohydrate

Table 4.2

Ability of D-mannosamine and D-galactose to Inhibit the Autorosetting of Different Congenic and Recombinant Strains of Thymocytes

Thymocyte Strain a)	Haplotype K $\frac{I}{ABJEC}$ S G D	% Rosette-Forming Cells a)		
		Control	D-mannosamine	D-galactose
B10.D2	d ddddd d d d	60 ± 2.4	55 ± 3.3	57 ± 3.7
B10.G	q qqqqq q q q	56 ± 3.4	[15 ± 2.9] b)	55 ± 3.2
B10.T(6R)	q qqqqq q ? d	55 ± 2.9	54 ± 2.8	52 ± 2.9
B10.A(2R)	k kkkkd d ? b	55 ± 3.8	[17 ± 4.2]	53 ± 3.3
B10	b bbbbb b b b	49 ± 2.9	[18 ± 2.2]	46 ± 4.4
SJL/J	s sssss s s s	20 ± 1.7	18 ± 2.3	[9 ± 3.1]
ATH	s sssss s s d	39 ± 2.8	35 ± 4.0	36 ± 2.6
B10.BR	k kkkkk k k k	61 ± 3.8	54 ± 4.6	59 ± 3.9

a) Thymocytes preincubated for 60 min at 4°C with sugar (20 mg/ml) prior to rosetting with autologous erythrocytes.

b) Results expressed as % rosetting cells ± standard deviation of three determinations. Values which represent significant inhibition are enclosed.

Table 4.3

Effect of Different Enzymic or Chemical Treatments on the Ability of CBA/H Thymocytes and Erythrocytes to Autorosette

Enzyme or Chemical	Cells treated[a]	
	Erythrocytes	Thymocytes
Nil	31 ± 3.1[b]	33 ± 2.8
Trypsin	19 ± 1.5	20 ± 2.4
Bromelin	2 ± 1.1	2 ± 1.2
Pronase	3 ± 1.3	5 ± 1.2
Papain	2 ± 1.2	6 ± 1.2
α-galactosidase	30 ± 2.2	29 ± 2.7
β-galactosidase	10 ± 2.7	28 ± 3.1
Neuraminidase	19 ± 2.6	30 ± 2.3
α-mannosidase	12 ± 1.6	28 ± 1.6
Mixed glycosidases	3 ± 1.4	27 ± 2.9
Sodium Periodate (10^{-4}M)	1 ± 1.2	31 ± 2.5
Sodium Periodate (10^{-5}M)	31 ± 2.4	32 ± 2.9

a) Erythrocytes or thymocytes were treated with the appropriate enzyme or chemical prior to rosetting with autologous thymocytes or erythrocytes.

b) Results expressed as % autorosetting cells ± standard deviations of three determinations.

structure on erythrocytes.

The sugar inhibition data in Table 4.1 suggested subtle differences in the carbohydrate structure being recognized by thymocytes from mouse strains of different H-2 haplotype. As an extension of this study, the effect of different exoglycosidases on the rosetting behaviour of thymocytes and erythrocytes from five mouse strains was examined (Table 4.4). None of the glycosidase treatments affected the autorosetting ability of thymocytes from the five strains. On the other hand, the glycosidases had strain-specific effects on the rosetting potential of the erythrocytes. Thus, β-galactosidase substantially reduced the rosetting of CBA/H ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) erythrocytes whereas α-galactosidase destroyed the acceptor of SJL/J ($H\text{-}2^s$), B10.G ($H\text{-}2^q$) and C57BL/6 ($H\text{-}2^b$) erythrocytes. Also, α-mannosidase had a selective effect on rosetting destroying the acceptor site on CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) erythrocytes. Neuraminidase was the least effective glycosidase, partially destroying acceptor sites on CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) and SJL/J ($H\text{-}2^s$) red cells. Although not shown in Table 4.4, the acceptor on erythrocytes from the B10.D2-$H\text{-}2^{dm1}$ mutant strain was susceptible to the same glycosidases as the acceptor on wild-type B10.D2 ($H\text{-}2^d$) erythrocytes even though the mutant exhibited a slightly different sugar inhibition behaviour (Table 4.1). It should be noted that the specificity of the glycosidases was confirmed by sugar blocking experiments. The effect of β-galactosidase on erythrocytes was blocked by lactose (40 mg/ml), the effect of α-galactosidase by melibiose (40 mg/ml) and the action of α-mannosidase by mannose (40 mg/ml).

As with the sugar inhibition studies, the genetic basis of the effects of the glycosidases was examined using erythrocytes from different H-2 congenic and recombinant mice (Table 4.5). It was found that the effects of the glycosidases mapped to the D-region of the MHC. Thus, with α-galacto-

Table 4.4

Effect of Different Glycosidase Treatments on Autorosetting of Thymocytes and Erythrocytes from Different Mouse Strains

Enzyme used	Cells treated [a]	Mouse Strains				
		CBA/H (k) [c]	BALB/c (d)	SJL/J (s)	B10.G (q)	C57BL/6 (b)
Nil	-	30 ± 2.9	42 ± 4.7	18 ± 2.2	53 ± 4.8	46 ± 4.6
β-galactosidase	Erythrocytes	[7 ± 2.9] [b]	[10 ± 2.5]	20 ± 1.9	48 ± 3.8	42 ± 4.6
β-galactosidase	Thymocytes	31 ± 2.8	39 ± 3.5	19 ± 1.9	50 ± 3.2	48 ± 3.1
α-galactosidase	Erythrocytes	28 ± 3.1	36 ± 2.9	[3 ± 1.9]	[27 ± 4.7]	[29 ± 3.4]
α-galactosidase	Thymocytes	31 ± 3.1	39 ± 3.3	19 ± 1.8	53 ± 4.1	47 ± 3.9
α-mannosidase	Erythrocytes	[11 ± 1.7]	[10 ± 2.6]	18 ± 2.3	55 ± 5.5	[31 ± 4.1]
α-mannosidase	Thymocytes	31 ± 2.9	37 ± 4.2	19 ± 2.0	53 ± 5.1	48 ± 3.2
Neuraminidase	Erythrocytes	[20 ± 2.6]	[31 ± 2.8]	[13 ± 3.4]	48 ± 4.7	43 ± 3.6
Neuraminidase	Thymocytes	30 ± 3.1	40 ± 3.2	20 ± 2.2	54 ± 4.2	47 ± 4.6

a) Erythrocytes or thymocytes were treated with the appropriate enzyme prior to rosetting with autologous thymocytes or erythrocytes.

b) Results expressed as % autorosetting cells ± standard deviations of three determinations. Values which represent significant effect are enclosed.

Table 4.5

Effect of Different Glycosidase Treatments on Autorosetting Ability of Erythrocytes from Different Mouse Strains

Strain	Haplotype K I/ABJEC S G D	Glycosidase Treatment [a]			
		Nil	α- galactosidase	β-galactosidase	α-mannosidase
B10.D2	d ddddd d d d	57 ± 4.3	52 ± 2.6	34 ± 4.1 [b]	36 ± 2.4
B10.G	q qqqqq q q q	55 ± 3.7	33 ± 4.1	56 ± 3.6	50 ± 3.5
B10.T(6R)	q qqqqq q ? d	58 ± 3.9	52 ± 3.9	37 ± 2.8	35 ± 2.7
B10.A(2R)	k kkkkd d ? b	54 ± 2.2	31 ± 2.8	46 ± 3.3	29 ± 3.8
B10	b bbbbb b b b	48 ± 2.9	25 ± 3.2	45 ± 3.8	28 ± 3.4
SJL/J	s sssss s s s	18 ± 2.1	6 ± 2.6	21 ± 1.7	19 ± 2.9
ATH	s sssss s s d	37 ± 3.7	40 ± 1.7	4 ± 2.2	6 ± 1.9
B10.BR	k kkkkk k k k	59 ± 4.1	54 ± 4.4	34 ± 3.6	36 ± 3.9

a) Erythrocytes were treated with the appropriate glycosidase for 30 min at 37°C prior to rosetting with autologous thymocytes.

b) Results expressed as % autorosetting cells ± standard deviation of three determinations.

sidase the effect on erythrocytes from b, q and s haplotypes was mapped to the D-end by the recombinants B10.A(2R), B10.T(6R) and A.TH, respectively. Similarly, the influence of β-galactosidase on the rosetting potential of d haplotype erythrocytes was mapped to the D region by the recombinants B10.T(6R), B10.A(2R) and A.TH. Finally, the effect of α-mannosidase on d haplotype rosetting mapped to the D-region on the basis of the recombinants B10.T(6R) and A.TH. There was insufficient information to map the effects of α-mannosidase on b and k haplotypes and β-galactosidase on k haplotype to the D-region, although the data available was consistent with this possibility.

4.3 Discussion

The experiments described in this chapter attempted to analyse the chemical basis of the H-2-controlled interaction between thymocytes and autologous erythrocytes. It was found that rosetting was inhibited, in a strain-specific manner, by a range of simple sugars (Table 4.1), an observation that suggested that rosetting involved carbohydrate recognition. Subsequent treatment of either thymocytes or erythrocytes with specific exoglycosidases demonstrated that thymocytes, in fact, recognized a carbohydrate structure on erythrocytes (Tables 4.3 and 4.4). The extreme susceptibility of the erythrocyte acceptor to periodate oxidation (Table 4.3) was consistent with this conclusion. Furthermore, strain differences in the sugar inhibition and glycosidase effects mapped to the H-2L/H-2D region of the murine MHC (Tables 4.2 and 4.5), confirming the H-2 controlled nature of the roesetting phenomenon. On the other hand, both the thymocyte receptor and erythrocyte acceptor were destroyed by proteases (Table 4.3), a finding previously reported by Kolb (1978). This result suggests that the thymocyte receptor is probably a protein and, combined with the sugar inhibition and glycosidase findings, the target structure on erythrocytes

the carbohydrate portion of possibly a glycoprotein.

It could be argued that the enzymic and chemical treatments that modified the rosetting behaviour of thymocytes and erythrocytes are indirectly, rather than directly, affecting cell surface structures. This could particularly be the case with periodate oxidation and neuraminidase treatment where the charge on cells is modified. This possibility seems unlikely, however, as the sugar inhibition and glycosidase results complement each other and show H-2-linked strain differences. It should also be noted that the specificity of the glycosidases was confirmed by sugar blocking experiments. The effect of β-galactosidase on erythrocyte was blocked by lactose (40 mg/ml), the effect of α-galactosidase by melibiose (40 mg/ml) and the action of α-mannosidase by D-mannose (40 mg/ml).

Table 4.6 summarises the sugars and glycosidases that affected the rosetting of different H-2 haplotypes. Based on this information it is possible to propose a tentative structure of the carbohydrate moiety recognized on erythrocytes. The monosaccharides D-galactosamine, D-mannose and N-aceyl-neuraminic acid inhibited the rosetting of all mouse strains tested. This result suggests that these sugars, or close analogues, play a prominent role in the acceptor structure. The glycosidase results support this contention as in all strains the target structure was susceptible to either α- or β-galactosidase and, in some strains, it was affected by α-mannosidase and neuraminidase. Furthermore, since these glycosidases only cleave terminal monosaccharides, the carbohydrate acceptor must be a branched chain structure containing terminal D-galactose, D-mannose and sialic acid. On the other hand, differences in the linkage of these three monosaccharides occur between different haplotypes. Thus, the galactosidase results indicate that D-galactose is α-linked in q, s and b haplotypes and β-linked in k and d haplotypes. Similarly, D-mannose is α-linked in k, d and b haplotypes and may be β-linked in q and s haplotypes, although a

Table 4.6

Summary of sugar and glycosidase Treatments that Inhibit Rosetting of Thymocytes of Different Haplotype

	Haplotype				
	K	d	q	s	b
Sugar					
D-galactosamine	+	+	+	+	+
D-galactose	-	-	-	+	-
D-mannose	+	+	+	+	+
D-mannosamine	-	-	+	-	+
N-acetylneuraminic acid	+	+	+	+	+
raffinose	+	+	+	+	+
maltose	+	+	+	+	+
mannan	+	+	+	+	+
Glycosidase					
β-galactosidase	+	+	-	-	-
α-galactosidase	-	-	+	+	+
α-mannosidase	+	+	-	-	+
neuraminidase	+	+	-	+	-

\+ indicates a significant effect

\- indicates no effect

β-mannosidase was not available to test this possibility. Furthermore, the strain-specific blocking by D-galactose and D-mannosamine suggests that strains can differ not only in the αor β linkage but in the point of linkage in the carbon ring of these sugars, e.g., only s haplotype was inhibited by D-galactose even though the rosetting of s, q and b haplotypes was affected by α-galactosidase.

Although the galactosidase studies implicate either α- or β-linked D-galactose in the acceptor site, D-galactosamine rather than D-galactose was usually the inhibitory sugar (Table 4.1). It is unlikely that D-galactosamine is the terminating sugar as the α- and β galactosidase preparations are free of galactosaminidase. A more likely explanation is that, since the thymocyte receptor appears to accommodate several sugars, D-galactosamine more effectively masks the binding site of the receptor than D-galactose. Similarly, the inhibition of rosetting by raffinose (D-Gal-α1-6-D-Glu-α1-2-D-Fru) but not by stachyose (D-Gal-α1-6-D- Gal-α1—6-D-Glu-α1-2-D-Fru) could be explained in this way. Finally, the blocking of rosetting by mannan, a large mol. wt. polymer of D-mannose, supports D-mannose involvement in the acceptor. In contrast, blocking by maltose (D-Glu-α1-4-D-Glu) was unexpected. Presumably this disaccharide mimics the core of the branched chain structure being recognized.

Collectively, the sugar inhibition and glycosidase results indicate that autorosetting involves H-2 controlled receptors on thymocytes recognizing terminal D-galactose, D-mannose and sialic acid residues on a branched chain carbohydrate structure on erythrocytes. Mouse strains of different H-2 haplotypes express carbohydrate structures that differ in the linkage of these three terminal sugars. Until recently there was little evidence for carbohydrate H-2 antigens, but recent studies with monoclonal anti-H-2 antibodies now suggest that carbohydrate H-2 antigenic specificities do indeed exist (O'Neill et al 1981, O'Neill

and Parish 1981). Such a conclusion has important theoretical implications and these will be discussed in detail in chapter 7.

Summary

Rosetting between thymocytes and autologous erythrocytes is mediated by receptors on thymocytes that primarily recognize self H-2L/H-2D molecules on erythrocytes. This chapter describes preliminary attempts to chemically characterize the receptor and acceptor molecules involved in this H-2-controlled interaction. On the basis of sugar inhibition studies and the sensitivity of the receptors and acceptors to protease and glycosidase treatments it appears that a protein receptor on thymocytes recognizes the carbohydrate portion of a glycoprotein on erythrocytes. Furthermore, the thymocyte receptor appears to recognize terminal D-galactose, D-mannose and sialic acid residues on a branched chain carbohydrate structure on erythrocytes, mouse strains of different H-2 haplotype expressing carbohydrate structures that differ in the linkage of these three terminal sugars. These findings indicate that H-2-controlled carbohydrate-protein interactions can occur between cells, a conclusion with important theoretical implications.

Chapter 5

Characterisation of Receptors and Acceptors Involved in Autorosetting

Table of Contents

5.1 Introduction

There are several reports that subpopulations of murine lymphocytes bind autologous erythrocytes, a phenomenon termed autorosetting (Micklem and Asfi 1971, Charriere and Bach 1975, Kolb 1977, Steele and Cunningham 1980). Recently three different laboratories demonstrated that this interaction is H-2 controlled, lymphocytes preferentially binding H-2 compatible erythrocytes (chapter 3, Primi et al 1979, Charriere et al 1980). Furthermore, detailed genetic analysis described in chapter 3 demonstrated that lymphocytes carry H-2L/H-2D region controlled receptors which preferentially recognize self H-2L/H-2D molecules on the erythrocyte surface. Based on these observations, the autorosetting phenomenon represents an ideal opportunity to analyse the molecular nature of a MHC-controlled cell-cell interaction.

Preliminary studies described in chapter 4 indicate that the autorosetting receptor is a protein which recognizes the carbohydrate portion of a glycoprotein on erythrocytes. This conclusion was based on two experimental approaches. First, certain monosaccharides and oligosaccharides inhibited autorosetting, the pattern of inhibitory sugars differing between H-2 haplotypes, an effect which mapped to the D-end of the H-2 complex. Second, both the thymocyte receptor and erythrocyte acceptor were protease sensitive. However, the erythrocyte acceptor also was destroyed by certain exoglycosidases, the glycosidase susceptibility of the acceptor being dependent upon the H-2 haplotype of the erythrocyte. Furthermore, as with the sugar inhibition studies, the differences in glycosidase susceptibility mapped to the D-end of the MHC. On the basis of these observations it was proposed that the thymocyte receptor recognizes terminal D-galactose, D-mannose and sialic acid residues of a branched chain carbohydrate structure on erythrocytes, mouse strains of different H-2 haplotype expressing carbohydrate structures that differ in

the linkage of these three terminal sugars.

This chapter describes attempts to isolate and characterize the receptor and acceptor molecules from detergent lysates of thymocytes and erythrocytes. The study supports the concept that autorosetting is mediated by a H-2L/H-2D region controlled lectin that recognizes a protein-bound carbohydrate structure on erythrocytes.

5.2 Results

5.2.1 Detection of Haemagglutinin in Detergent Lysates of Lymphoid Cells

Since there is ample evidence that murine thymocytes and peripheral T and B lymphocytes carry receptors for autologous erythrocytes, it seems possible that detergent lysates of lymphoid cells may contain haemagglutinating activity. To test this possibility, advantage was taken of the recent observation that NP-40 lysates of lymphoid cells can be depleted of NP-40 with detergent binding beads (Parish et al 1980). The resultant lysates contain enough residual detergent to keep membrane components in solution, but insufficient detergent to lyse intact cells.

In fact, when NP-40 lysates of BALB/c thymocytes were depleted of detergent in this manner, the lysates strongly haemagglutinated BALB/c erythrocytes to a high dilution, i.e., titre of 1/128 (Table 5.1). In contrast, spleen cell lysates had a much lower haemagglutinating activity (titre of 1/4) and no haemagglutinin could be detected in lymph node or bone marrow lysates. This result correlates with both the prevalence and avidity of the autorosetting cells in these different lymphoid organs, i.e., the autorosetting frequency is thymus > spleen > lymph node > bone marrow (Micklem and Asfi 1971, Charriere and Bach 1975, chapter 3), and peripheral lymphocytes appear to bind fewer red cells then thymcoytes. It should be noted, however, that the cell lysates used could contain both membrane-associated and cytoplasmic haemagglutinins.

However, when thymocytes were separated into cytoplasmic and microsomal fractions all haemagglutinating activity was recovered in the microsomal fraction, implying that the haemagglutinin is membrane-bound (Table 5.1).

5.2.2 Specificity of Thymocyte Haemagglutinin

The next series of experiments tested the ability of a BALB/c thymocyte lysate to haemagglutinate syngeneic, allogeneic and xenogeneic erythrocytes. It was found (Table 5.2) that the lysate haemagglutinated all mouse erythrocytes tested. Furthermore, the lysate agglutinated rat but not sheep or chicken erythrocytes. In fact, the lysate gave a higher haemagglutination titre on rat than on mouse red cells, i.e., titre of 1/2048 compared with titre of 1/96 - 1/256. This was a surprising result, even though murine lymphocytes can bind rat erythrocytes apparently via the receptors that mediate autorosetting (chapter 3). The result could be due to either rat erythrocytes being intrinsically more readily haemagglutinated than mouse red cells or an additional haem-agglutinin in the thymocyte lysate that is specific for rat red cells.

Although red cells from all mouse strains tested were haemagglutinated by the BALB/c thymocyte lysate, it was noted that the lysate consistently gave slightly higher haemagglutination titres on some red cells than others, e.g., the BALB/c lysate gave a titre of 1/96 on CBA/H and 1/256 on C57BL/6 red cells (Table 5.2). An analysis of this effect is presented in more detail in Table 5.3. It can be seen that background (non-H-2) genes influenced the ability of different erythrocytes to be haemagglutinated. For example, B10.BR and CBA/H thymcocyte lysates gave higher haemagglutination titres on B10.BR (1/192 - 1/256) than on CBA/H (1/64 - 1/96) red cells. It is noteworthy that these background effects correspond with the ability of different red cells to rosette with murine spleen cells and thymocytes (chapter 3).

Table 5.1

Distribution of Haemagglutinin in Lymphoid Organs of BALB/c Mice

Lymphoid organ	Haemagglutination Titre on BALB/c erythrocytes
Thymus	128 ± 9 c)
Spleen	4 ± 2
Lymph Node	<2
Bone Marrow	<2
Thymocytes (cytoplasmic fraction)	<2
Thymocytes (microsomal fraction)	128

c) Results expressed as haemagglutination titre ± standard errors of three determinations.

Table 5.2

Ability of BALB/c Thymocyte Lysate to Haemagglutinate Different Erythrocytes

Erythrocytes	H-2 haplotype	Haemagglutination Titre
BALB/c	d	128 ± 7 c)
DBA/2	d	128 ± 8
CBA/H	k	96 ± 6
B10.BR	k	192 ± 9
SJL/J	s	128 ± 9
C57BL/6	b	256 ± 10
B10.G	q	192 ± 7
Rat (Lewis)	-	2048 ± 14
Sheep	-	<2
Chicken	-	<2

c) Results expressed as haemagglutination titre ± standard errors of three determinations.

Table 5.3

Influence of Erythrocyte Background on Haemagglutinating Activity of Thymocyte Lysates

Lysate Source	Erythrocyte Source	Haemagglutination Titre
B10.BR (H-2^k)	B10.BR	256 ± 11 c)
B10.BR	CBA/H	96 ± 7
CBA/H (H-2^k)	CBA/H	64 ± 6
CBA/H	B10.BR	192 ± 9
B10.D2 (H-2^d)	B10.D2	256 ± 10
B10.D2	BALB/c	96 ± 5
BALB/c (H-2^d)	BALB/c	96 ± 8
BALB/c	B10.D2	192 ± 9
B10.G (H-2^q)	B10.G	192 ± 9
B10.G	DBA/1	96 ± 8
DBA/1 (H-2^q)	DBA/1	96 ± 6
DBA/1	B10.G	256 ± 8

c) Results expressed as haemagglutination titre ± standard error of three determinations.

5.2.3 Strain Differences in Haemagglutinating Activity

The data presented in Table 5.3 indicate that thymocyte lysates from six different mouse strains (i.e., B10.BR, CBA/H, B10.D2, BALB/c, B10.G and DBA/1) gave comparable haemagglutination titres when tested on B10 background erythrocytes. However, analysis of additional strains revealed that lysates of C57BL/6 thymocytes consistently gave low haemagglutinating titres. Table 5.4 presents typical data in which a C57BL/6 lysate gave a haemagglutination titre of 1/32 compared with titres of 1/192 - 1/256 for lysates from other strains. Furthermore, the C57BL/6 lysate gave low haemagglutination titres on autologous, syngeneic and allogeneic erythrocytes (data not shown) implying that this effect was a property of the thymocyte lysate rather than the red cells.

It appears (Table 5.4) that the low haemagglutinating activity of C57BL/6 lysates is probably a property of the H-2^b haplotype as thymocyte lysates from strains with different H-2 haplotypes but virtually the same genetic background as C57BL/6 exhibited high haemagglutination titres, i.e., B10.G (H-2^q), B10.D2 (H-2^d) and B10.BR (H-2^k). In fact, the effect was directly mapped to the D-end of the H-2 complex by the two H-2 recombinant strains B10.A and B10.A(2R), as B10.A (K^k, D^d) lysates had high haemagglutinating activity (1/256), whereas B10.A(2R) (K^k, D^b) lysates exhibited low haemagglutination titres (1/24) (Table 5.4).

Autorosetting receptors on lymphocytes interact with a carbohydrate structure on erythrocytes that is destroyed by periodate (chapter 4). It was thought, therefore, that if the autorosetting receptors and haemagglutinin are the same molecule, detergent lysis of lymphoid cells may release carbohydrate structures that neutralise the haemagglutinin. To overcome this problem, thymocytes were periodate-treated prior to solubilisation with NP-40. This treatment, in fact, restored the haemagglutinating activity of C57BL/6 and B10.A(2R) lysates to normal levels but did not

Table 5.4

Haemagglutination Activity of Lysates from Untreated or Periodate-Treated Thymocytes

Lysate Source	H-2 haplotype	Haemagglutination Titre	
		Untreated	Periodate-treated
C57BL/6	b	32 ± 5 [c)]	256 ± 10
B10.A(2R)	h2	24 ± 4	384 ± 14
B10.A(SgSn)	a	256 ± 14	384 ± 11
B10.G	q	192 ± 11	192 ± 11
B10.D2	d	256 ± 10	192 ± 14
B10.BR	k	256 ± 8	256 ± 11
SJL/J	s	192 ± 7	256 ± 12

c) Results expressed as haemagglutination titre ± standard error of three determinations. All thymocyte lysates tested on C57BL/6 erythrocytes.

augment the haemagglutination titres of thymocyte lysates from other strains (Table 5.4). The periodate treatment appeared to be modifying carbohydrate rather than protein structures on thymocytes as mixed glycosidase treatment of thymocytes also restored the haemagglutinating activity of C57BL/6 lysates (data not shown). It should be noted, however, that periodate treatment did not augment the haemagglutination titres of spleen, lymph node and bone marrow lysates (Table 5.1).

5.2.4 Analysis of Haemagglutinin in H-2^{dm2} Mutant Mouse

In chapter 3, it was demonstrated that lymphocytes from BALB/c-H-2^{dm2} mutant mice, a H-2L deletion strain (McKenzie *et al* 1977, Hansen *et al* 1977), were virtually unable to rosette with autologous, allogeneic or xenogeneic erythrocytes. It was important, therefore, to assess the haemagglutinating potential of the thymocyte lysate from this mutant mouse (Table 5.5). It was found that the mutant lysate had a much lower haemagglutination titre (1/12) than the thymocyte lysate prepared from the wild-type BALB/c strain (titre of 1/96). This result is certainly consistent with the haemagglutinin and autorosetting receptors being the same molecule.

It is of interest to note that the mutant thymocytes were not completely devoid of haemagglutinating activity. The residual activity may be derived from the residual population of autorosetting cells present in the thymus of this mutant (chapter 3).

5.2.5 Sugar Inhibition of Thymocyte Haemagglutinin

Earlier studies (chapter 4) demonstrated that the autorosetting receptors on thymocytes recognize a carbohydrate structure on erythrocytes. If the haemagglutinin described above represents the autorosetting receptor, it would be expected that the same sugars that inhibit autorosetting would inhibit haemagglutination.

Table 5.5

Ability of BALB/c and BALB/c-H-2^{dm2} Thymocyte Lysates to Agglutinate BALB/c Erythrocytes

Thymocyte lysate	Haemaagglutination Titre
BALB/c	96 ± 9 [c]
BALB/c-H-2^{dm2}	12 ± 7

c) Results expressed as haemagglutination titre ± standard errors of three determinations.

A total of thirty monosaccharides and oligosaccharides were tested for their ability to inhibit haemagglutination. These were the same thirty sugars that were previously tested for inhibition of autorosetting (chapter 4). Of the thirty sugars screened, only eight inhibited haemagglutination, namely D-galactose, D-galactosamine, D-mannose, D-mannosamine, N-acetylneuraminic acid, maltose, raffinose and mannan (Table 5.6). These were the same eight sugars that inhibited autorosetting (chapter 4).

Six of the eight inhibitory sugars were equally inhibitory for thymocyte lysates from six different mouse strains (Table 5.6). However, these six sugars varied in their inhibitory activity being mannan > D-mannosamine > N-acetylneuraminic acid > mannose > maltose > raffinose. This point is more clearly illustrated in Fig. 5.1 which depicts the effect of different concentrations of the inhibitory sugars on the haemagglutination titre of a C57BL/6 thymocyte lysate.

It should be noted, however, that two of the inhibitory sugars, namely D-galactose and D-galactosamine, exhibited strain-specific inhibition (Table 5.6). Thus, D-galactose inhibited the haemagglutinin from SJL/J ($\underline{H\text{-}2}^{s}$) mice, an effect that appears to map to the $\underline{D}$-end of the $\underline{H\text{-}2}$ complex, as lysates from A.TH ($\underline{K}^{s}$, $\underline{D}^{d}$) mice were not inhibited by D-galactose (data not shown). Furthermore, D-galactose blocked haemagglutination by a lysate of B10.D2 (M504) mutant thymocytes, but did not inhibit a lysate from wild-type B10.D2 (Table 5.6). Since the B10.D2 (M504) strain represents a $\underline{H\text{-}2L}/\underline{H\text{-}2D}$ mutant (Morgan $\underline{et\ al}$ 1978), this result is also consistent with the haemagglutinin being under $\underline{H\text{-}2L/D}$ region control.

On the other hand D-galactosamine, although inhibiting the haemagglutinins of all mouse strains, was substantially less inhibitory for a C57BL/6 ($\underline{H\text{-}2}^{b}$) lysate (Table 5.6). This difference is more clearly depicted in Fig. 5.2, which shows the effect of different concentrations of D-galactosamine on haemagglutinating activity. Fig. 5.2 also maps

Table 5.6

Ability of Different Sugars to Inhibit the Haemagglutinating Activity of Different Thymocyte Lysate

Sugar inhibitor	Strain of origin of thymocyte lysate [a]					
	C57BL/6 (b) [b]	B10.BR (k)	B10.G (q)	SJL/J (s)	B10.D2 (d)	B10.D2 (M504) (dml)
Monosaccharides						
D-glucose	256 ± 10 [c]	192 ± 9	256 ± 10	192 ± 7	256 ± 12	256 ± 13
D-galactose	256 ± 11	192 ± 9	256 ± 11	64 ± 12	256 ± 10	96 ± 9
D-galactosamine	32 ± 7	4 ± 2	4 ± 3	4 ± 3	4 ± 4	8 ± 4
D-mannose	64 ± 6	64 ± 8	48 ± 7	64 ± 7	48 ± 5	64 ± 7
D-mannosamine	4 ± 3	4 ± 2	4 ± 2	6 ± 3	8 ± 3	8 ± 4
N-acetyl-neuraminic acid	32 ± 6	48 ± 5	48 ± 4	32 ± 6	48 ± 5	48 ± 6
Oligosaccharides						
maltose	64 ± 6	96 ± 8	64 ± 5	48 ± 6	64 ± 7	96 ± 6
raffinose	128 ± 7	128 ± 7	128 ± 6	96 ± 8	128 ± 9	96 ± 8
mannan	2 ± 2	2 ± 2	2 ± 1	2 ± 1	2 ± 1	2 ± 2

a) Thymocyte lysates preincubated with sugars (40 mg/ml) for 1 hr on ice prior to addition of C57BL/6 erythrocytes.

b) H-2 haplotype of mouse strains.

c) Results expressed as haemagglutination titre ± standard error of three determinations.

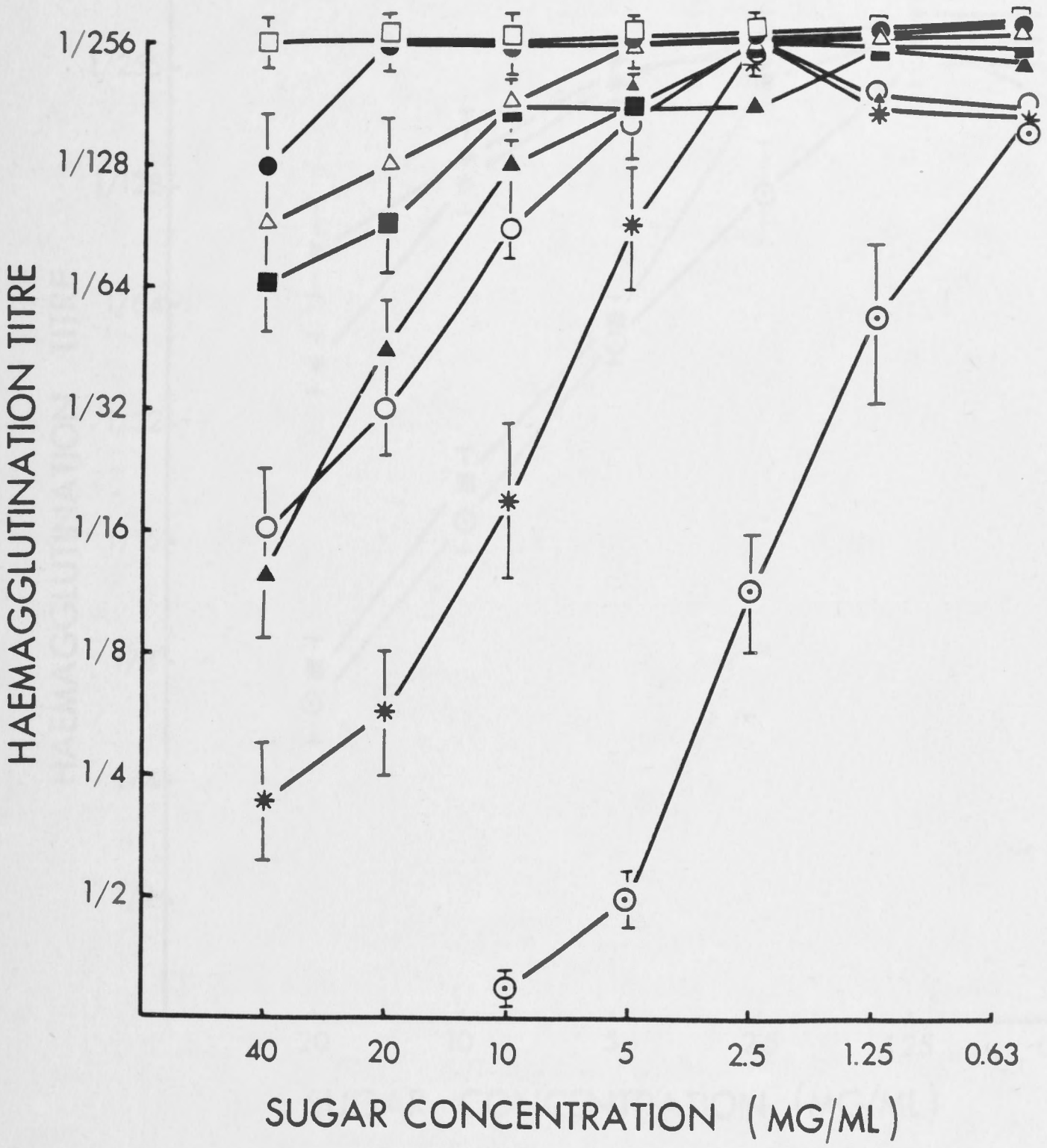

Fig. 5.1 Ability of different sugars to inhibit the haem-agglutination of C57BL/6 erythrocytes by a lysate of C57BL/6 thymocytes. Sugar inhibitors are: mannan (⊙), D-mannosamine (✳), N-acetyl-neuraminic acid (▲), D-galactosamine (○), D-mannose (■), maltose (△), raffinose (●) and D-glucose (□). Vertical bars represent standard deviations of means.

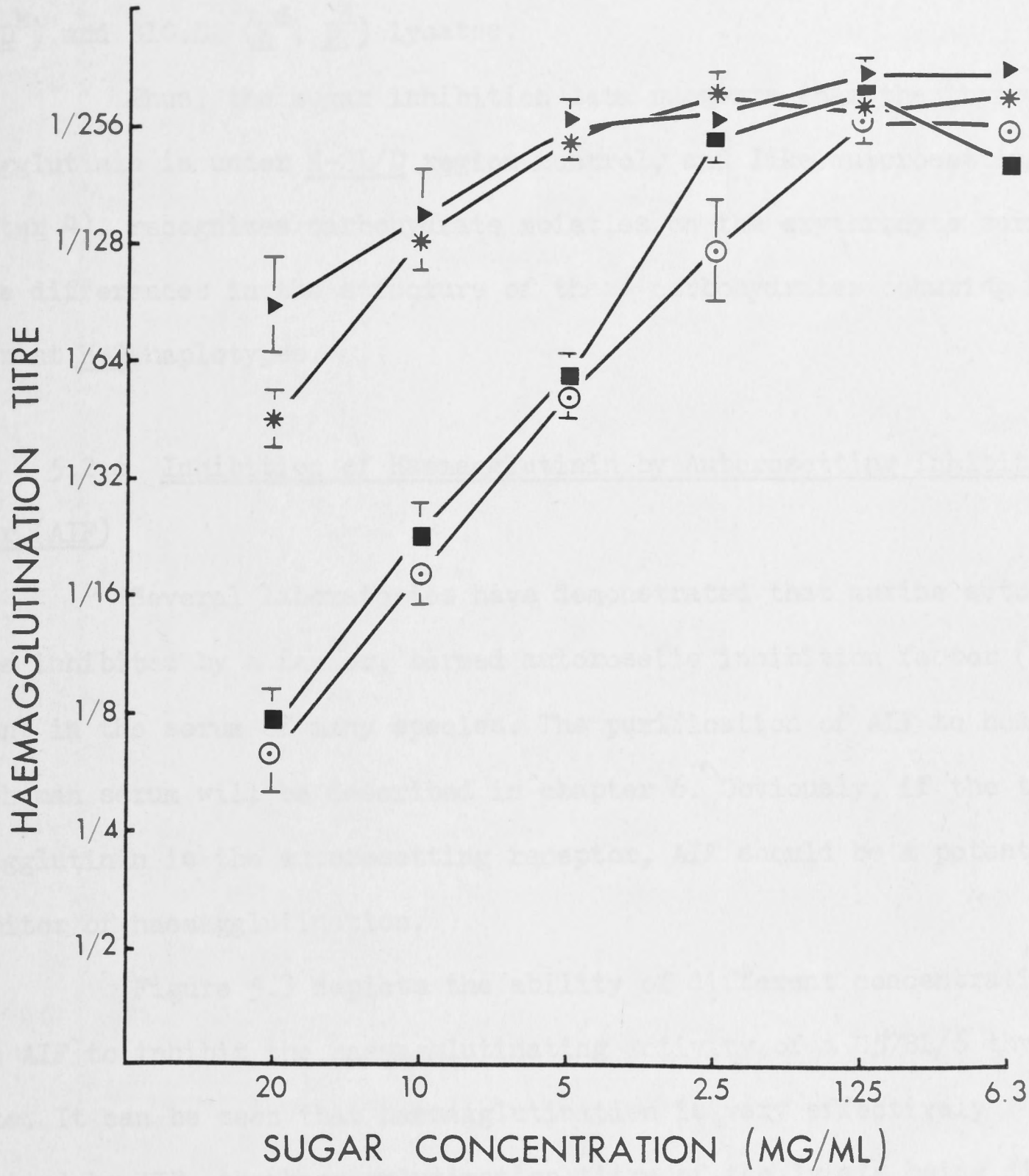

Fig. 5.2 Ability of different concentrations of D-galactosamine to inhibit the haemagglutination of C57BL/6 erythrocytes by thymocyte lysates from C57BL/6 (✳), B10.A(2R) (▶), B10.BR (⊙) and B10.D2 (■) mice. Vertical bars represent standard deviations of means. The haemagglutination titre of all the thymocyte lysates, in absence of the sugar inhibitor, was 1/256.

the difference to the D-end of the H-2 complex as C57BL/6 (K^b, D^b) and B10.A(2R) (K^k, D^b) lysates were inhibited less effectively than B10.BR (K^k, D^k) and B10.D2 (K^d, D^d) lysates.

Thus, the sugar inhibition data suggests that the thymocyte haemagglutinin is under H-2L/D region control, and like autorosetting (chapter 4), recognizes carbohydrate moieties on the erythrocyte surface; subtle differences in the structure of these carbohydrates occuring between different H-2 haplotypes.

5.2.6 Inhibition of Haemagglutinin by Autorosetting Inhibition Factor (AIF)

Several laboratories have demonstrated that murine autorosetting can be inhibited by a factor, termed autorosette inhibition factor (AIF), present in the serum of many species. The purification of AIF to homogeneity from human serum will be described in chapter 6. Obviously, if the thymocyte haemagglutinin is the autorosetting receptor, AIF should be a potent inhibitor of haemagglutination.

Figure 5.3 depicts the ability of different concentrations of human AIF to inhibit the haemagglutinating activity of a C57BL/6 thymocyte lysate. It can be seen that haemagglutination is very effectively inhibited by AIF, the haemagglutination titre of the lysate being directly proportional to the amount of AIF added.

5.2.7 Detection of Haemagglutinin Acceptor in Detergent Lysates of Erythrocytes

A procedure was devised for detecting the haemagglutinin acceptor on mouse erythrocytes. The procedure entailed preparing a NP-40 lysate of erythrocyte ghosts, depleting the lysate of NP-40 with detergent-binding beads and then assessing the ability of serial dilutions of the erythrocyte lysate to inhibit haemagglutination by a

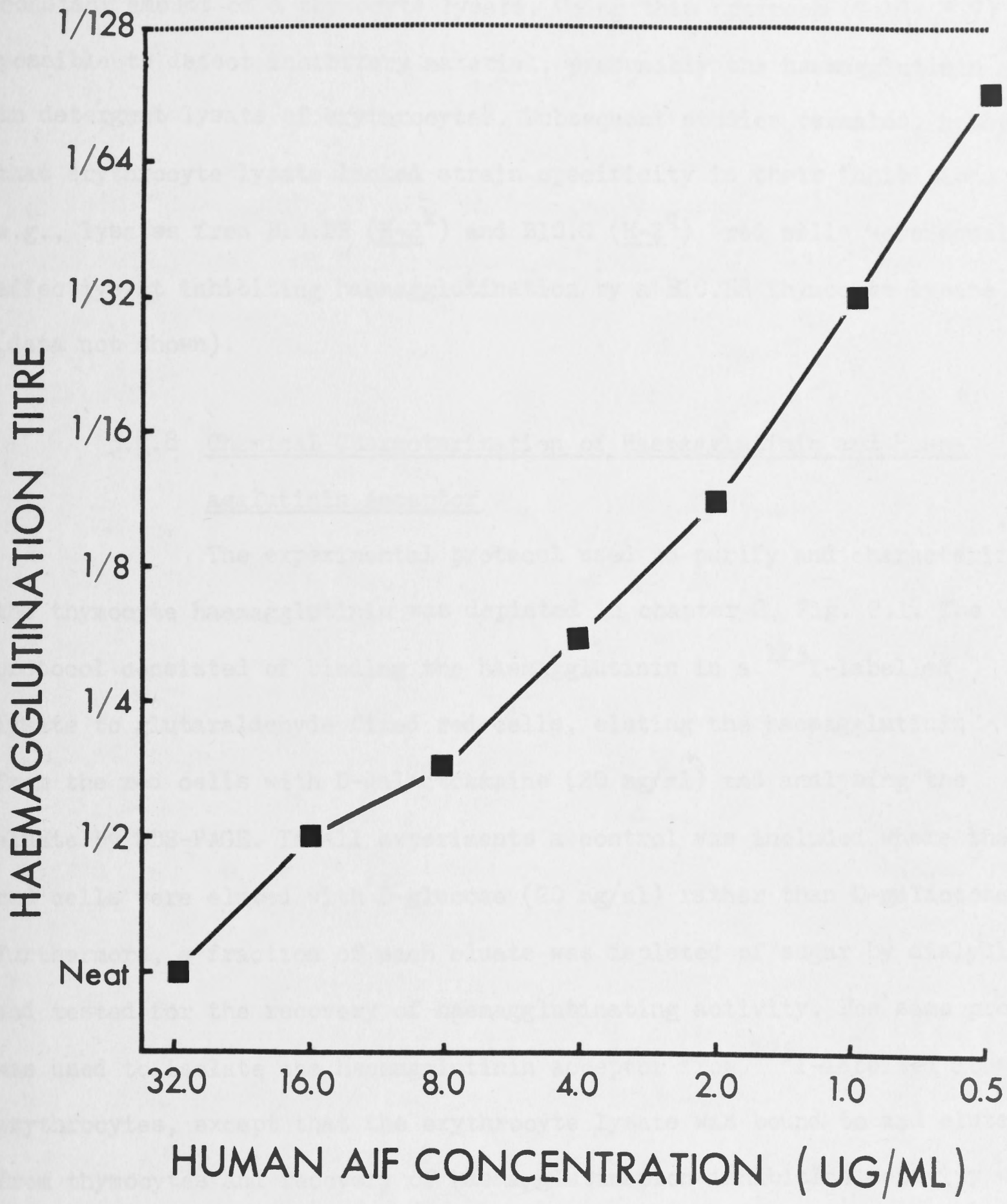

Fig. 5.3 Ability of different concentrations of pure human AIF to inhibit the haemagglutinating activity of a C57BL/6 thymocyte lysate. The haemagglutination titre of the thymocyte lysate in the absence of AIF, is shown by the dotted line.

constant amount of a thymocyte lysate. Using this approach (Table 5.7) it was possible to detect inhibitory material, presumably the haemagglutinin acceptor in detergent lysate of erythrocytes. Subsequent studies revealed, however, that erythrocyte lysate lacked strain specificity in their inhibition, e.g., lysates from B10.BR ($\underline{H\text{-}2}^{k}$) and B10.G ($\underline{H\text{-}2}^{q}$) red cells were equally effective at inhibiting haemagglutination by a B10.BR thymocyte lysate (data not shown).

5.2.8 Chemical Characterization of Haemagglutinin and Haemagglutinin Acceptor

The experimental protocol used to purify and characterize the thymocyte haemagglutinin was depicted in chapter 2, Fig. 2.1. The protocol consisted of binding the haemagglutinin in a ^{125}I-labelled lysate to glutaraldehyde fixed red cells, eluting the haemagglutinin from the red cells with D-galactosamine (20 mg/ml) and analysing the eluate by SDS-PAGE. In all experiments a control was included where the red cells were eluted with D-glucose (20 mg/ml) rather than D-galactosamine. Furthermore, a fraction of each eluate was depleted of sugar by dialysing and tested for the recovery of haemagglutinating activity. The same protocol was used to isolate the haemagglutinin acceptor from ^{125}I-labelled mouse erythrocytes, except that the erythrocyte lysate was bound to and eluted from thymocytes and recovery of haemagglutination-inhibition activity was determined.

Table 5.8 presents typical elution data for the haemagglutinin and haemagglutinin acceptor in terms of recovery of radioactivity and biological activity. It can be seen that the D-glucose eluates contained little radioactivity and no biological activity, whereas the D-galactosamine eluates contained substantial radioactivity and good recovery (75 - 100%) of either the haemagglutinin or haemagglutinin acceptor.

Table 5.7

Inhibition of Haemagglutinating Activity of BALB/c Thymocyte Lysate by BALB/c Erythrocyte Lysate

Dilution of thymocyte lysate a)	Inhibition titre of erythrocyte lysate
1/4	6 ± 2 c)
1/8	16 ± 4
1/16	48 ± 6
1/32	64 ± 6

a) Thymocyte lysates tested on BALB/c erythrocytes. Haemagglutination titre of lysate was 1/128.

c) Results expressed as inhibition titre ± standard error of three determinations.

Table 5.8

Isolation of Haemagglutinin and Haemagglutinin Acceptor:

Typical Elution Data

Preparation [a]	^{125}I c.p.m.	Titre [c]	
Thymocyte lysate (Haemagglutinin)			
D-glucose eluate	3,201	2	
D-galactosamine eluate	16,540	96	(128)
Erythrocyte lysate (Haemagglutinin acceptor)			
D-glucose eluate	2,107	2	
D-galactosamine eluate	10,546	16	(16)

a) Lysate from BALB/c mice. Thymocyte lysate bound and eluted from BALB/c erythrocytes, whereas erythrocyte lysate bound and eluted from BALB/c thymocytes.

c) Haemagglutination titre in the case of the thymocyte lysate and haemagglutination-inhibition titre in the case of the erythrocyte lysate. Values in brackets represent titres of lysates before fractionation.

Figures 5.4 and 5.5 present SDS-PAGE analysis of the haemagglutinin and haemagglutinin acceptor, respectively. In the case of the haemagglutinin, under non-reducing conditions the D-galactosamine eluate gave a single radioactive peak of molecular weight 63,000 daltons (± 2000 daltons based on three determinations) whereas under reducing conditions two radioactive peaks were seen with molecular weights of 54,000 ± 1000 and 45,000 ± 1000 daltons (Fig.5.4). These radioactive peaks were not detected in the D-glucose eluate. On the other hand, with the haemagglutinin acceptor the D-galactosamine eluate gave a radioactive peak of molecular weight 62,000 ± 2000 daltons both under reducing and non-reducing conditions, a peak that was not observed in the D-glucose eluate (Fig. 5.5).

5.3 Discussion

This chapter describes attempts to isolate and characterize the cell surface structures involved in the MHC-controlled interaction between autologous lymphocytes and erythrocytes. In initial attempts to isolate the autorosetting receptor(s) it was noted that NP-40 detergent lysates of thymocyte contained potent haemagglutinating activity for murine erythrocytes. Four lines of experimental evidence were obtained which suggested that this haemagglutinin was, indeed, the autorosetting receptor. First, the haemagglutinin had a similar lymphoid organ distribution to autorosetting cells, namely thymus > spleen > bone marrow, lymph node (Table 5.1). Furthermore, like autorosetting cells, the haemagglutinin lacked strain specificity, reacting with autologous, syngeneic and allogeneic erythrocytes (Tables 5.2 - 5.4). Second, the same eight sugars that inhibited autorosetting also inhibited the haemagglutinin (Table 5.6). Third, purified human AIF, a potent inhibitor of autorosetting, also very effectively inhibited the haemagglutinin (Fig. 5.3). Fourth, several results indicated that the

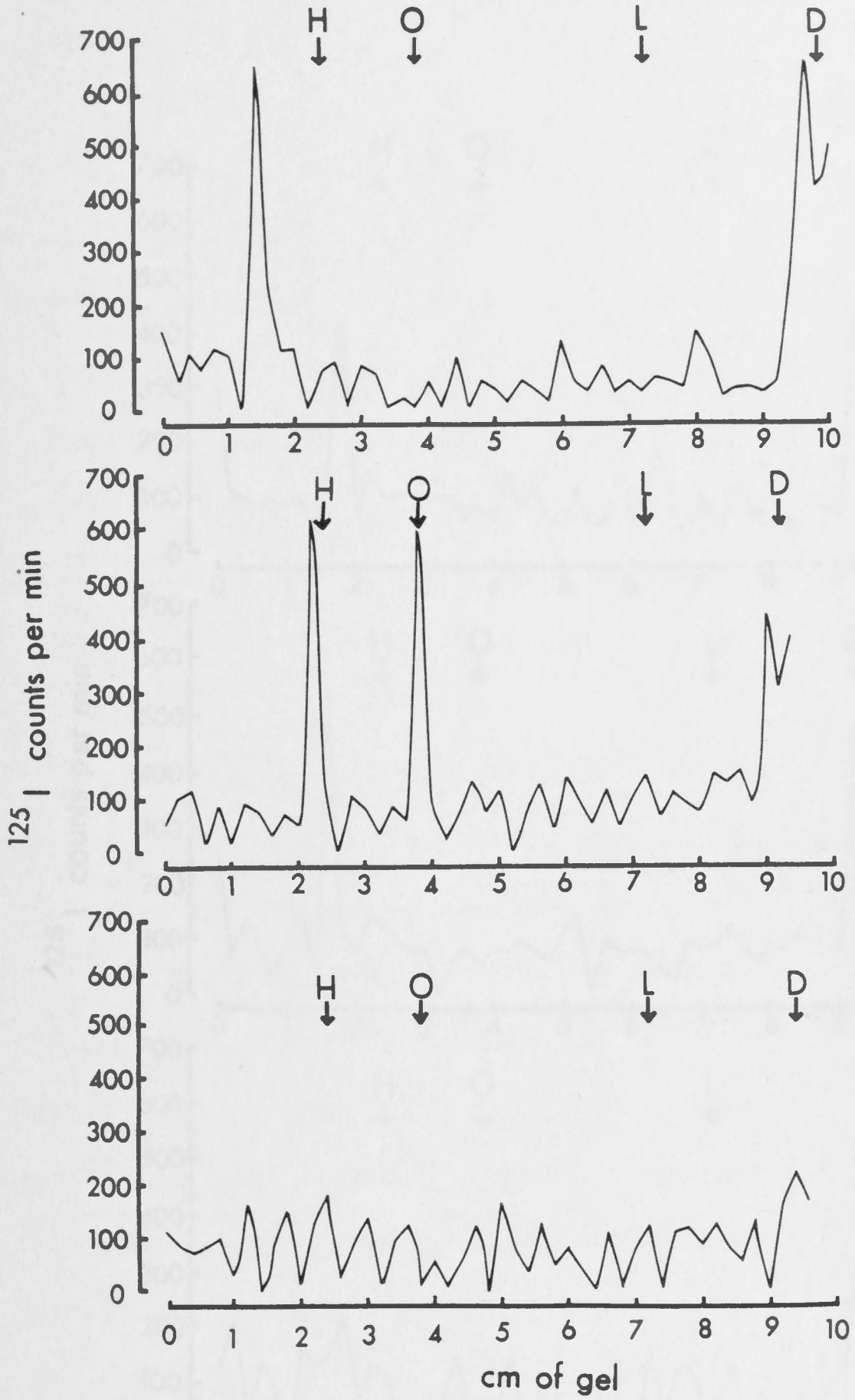

Fig. 5.4 SDS-polyacrylamide gel electrophoresis of ^{125}I-labelled BALB/c thymocyte receptor eluted from glutaraldehyde fixed BALB/c erythrocytes. The gel patterns represent (a) non-reduced D-galactosamine eluate, (b) reduced D-galactosamine eluate and (c) reduced D-glucose eluate. The migration distance of protein standards namely, IgG heavy chain (H), ovalbumin (O) and IgG light chain (L) and the dye marker (D) are indicated in each gel.

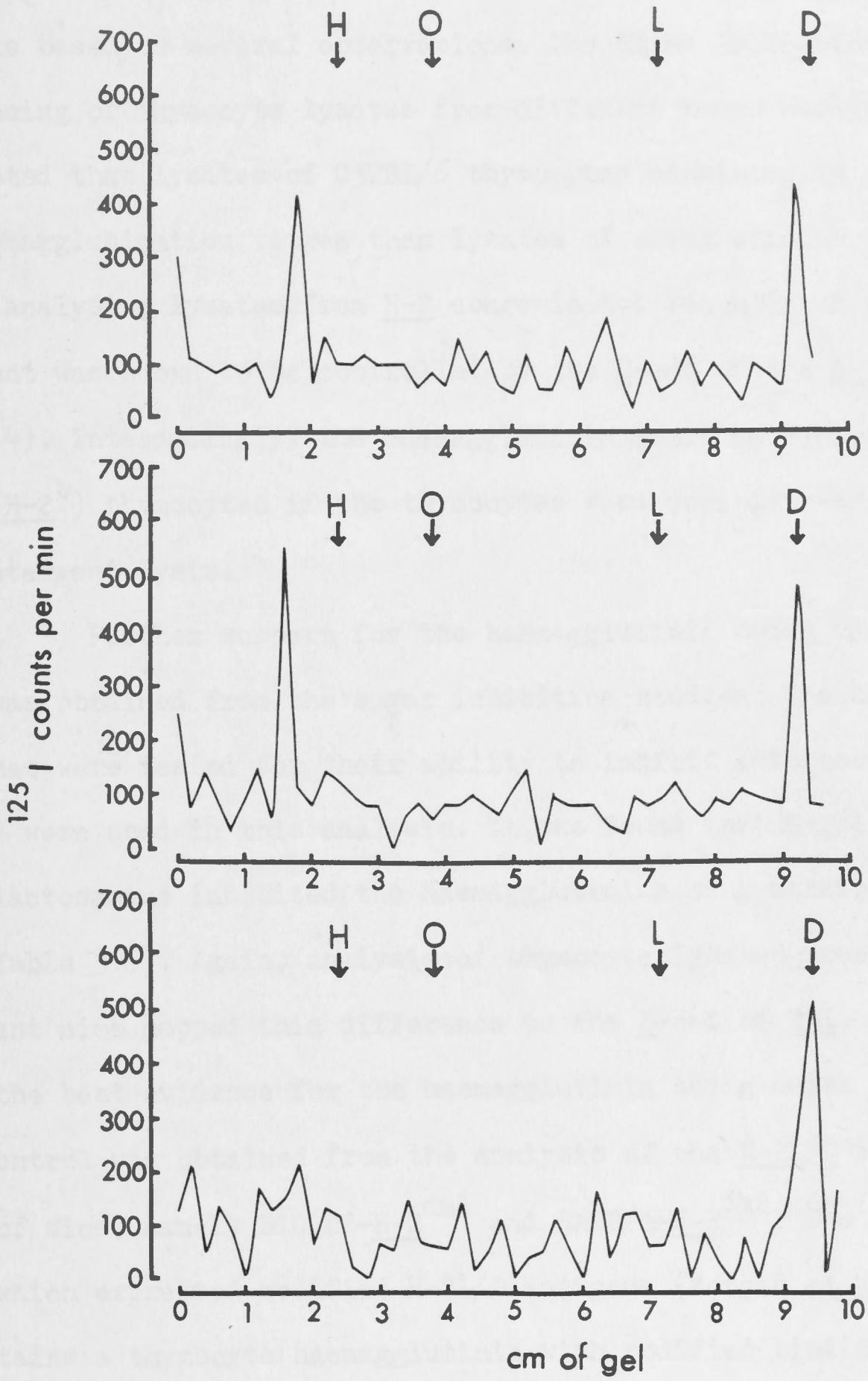

Fig. 5.5 SDS-polyacrylamide gel electrophoresis of ^{125}I-labelled BALB/c erythrocyte acceptor eluted from BALB/c thymocytes. The samples and protein standards are as in legend to Fig. 5.

haemagglutinin, like the autorosetting receptors, are controlled by the H-2L/D region of the H-2 complex.

The claim that the haemagglutinin is under H-2L/D region control is based on several observations. The first indication was in the screening of thymocyte lysates from different mouse strains where it was noted that lysates of C57BL/6 thymocytes consistently gave much lower haemagglutination titres than lysates of other strains (Table 5.4). By analysing lysates from H-2 congenic and recombinant mouse strains this effect was shown to be controlled by the D-end of the H-2 complex (Table 5.4). Interestingly, the haemagglutinin could be recovered from C57BL/6 ($H\text{-}2^{b}$) thymocytes if the thymocytes were periodate-treated before detergent lysis.

Further support for the haemagglutinin being under MHC control was obtained from the sugar inhibition studies. The same thirty sugars that were tested for their ability to inhibit autorosetting in chapter 4 were used in this analysis. It was found that D-galactose and D-galactosamine inhibited the haemagglutinins in a strain-specific manner (Table 5.6). Again, analysis of thymocyte lysates from H-2 recombinant mice mapped this difference to the D-end of H-2. However, perhaps the best evidence for the haemagglutinin being under H-2L/D region control was obtained from the analysis of the H-2L/D mutant strains of mice, namely B10.D2-$H\text{-}2^{dm1}$ and BALB/c-$H\text{-}2^{dm2}$. The B10.D2-$H\text{-}2^{dm1}$ mutant, which expressed modified H-2L/D antigens (Morgan et al 1978), also contains a thymocyte haemagglutinin with modified binding specificity, the mutant haemagglutinin, unlike the wild-type (B10.D2) haemagglutinin, being inhibited by D-galactose (Table 5.6). Similarly, thymocytes from the H-2L deletion mutant, BALB/c-$H\text{-}2^{dm2}$, that virtually lack autorosetting receptors (chapter 3), contained much less haemagglutinin than wild-type (BALB/c) thymocytes (Table 5.5). This finding strongly supports the

notion that the autorosetting receptors and the haemagglutinin represent the same molecule.

Although the same eight sugars inhibited the thymocyte haemagglutinin as inhibited autorosetting, there were some subtle differences between the two systems (summarised in Table 5.9). It can be seen that D-galactose, D-mannose, N-acetylneuraminic acid, raffinose, maltose and mannan gave comparable inhibition of haemagglutination and rosetting. In contrast, D-galactosamine was less inhibitory for b haplotype haemagglutination than b haplotype autorosetting and D-mannosamine exhibited haplotype specific inhibition of autorosetting but was equally inhibitory for all haemagglutinins. The explanation for these differences is uncertain but it seems possible that cell bound and solubilized receptors could differ slightly in their interaction with target structures.

As discussed in chapter 4, it could be argued that the sugars inhibit the haemagglutinin in a non-specific manner due to osmotic effects or due to impurities in the sugar preparations. There are several points which indicate that this explanation is unlikely: (i) only eight of the thirty sugars tested were inhibitory and these corresponded to the same sugars that inhibited autorosetting, (ii) the concentrations used to inhibit haemagglutination are similar to those used to establish the sugar specificity of numerous lectins in the past (Aswell and Morell 1977, Kieda et al 1978), and (iii) some sugars showed strain-specific inhibition that mapped to the H-2L/H-2D region.

Another important point that warrants discussion is the fact that the haemagglutinin, like autorosetting, lacks strain specificity in its activity, agglutinating both syngeneic and allogeneic red cells. If the autorosetting receptor has highest affinity for self H-2L/D molecules one might expect the detergent solubilised receptor

Table 5.9

Comparison of Sugars which Inhibit Thymocyte Haemagglutinin and Thymocyte Autorosetting

Sugar Inhibitor	H-2 haplotype of thymocytes					
	k	d	dml	q	s	b
D-galactose	- a) (-) b)	- (-)	+ (+)	- (-)	+ (+)	- (-)
D-galactosamine	++ (++)	++ (++)	++ (++)	++ (++)	++ (++)	+ (++)
D-mannose	+ (++)	+ (++)	+ (++)	+ (++)	+ (++)	+ (++)
D-mannosamine	++ (-)	++ (-)	++ (-)	++ (++)	++ (-)	++ (++)
N-acetyl-neuraminic acid	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
raffinose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
maltose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
mannan	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)

a) Inhibition of haemagglutinin.

b) Inhibition of autorosetting (chapter 5).

\+ designates strength of inhibition.

\- indicates no inhibition.

(haemagglutinin) to most effectively agglutinate H-2 compatible erythrocytes. However, gel filtration analysis of the haemagglutinin in detergent depleted lysates revealed that the molecule has a molecular weight of approx. 500,000 daltons and, therefore, must represent an aggregate of autorosetting receptors (D. B. Rylatt, personal communication). Thus the haemagglutinin could bind to red cells in a multipoint fashion and consequently similar to autorosetting, haemagglutinate allogeneic and xenogeneic (rat) red cells. In this context, the well characterized galactose binding lectin of hepatocytes consists of two subunits of molecular weight 48,000 and 40,000 daltons that readily form aggregates of 500,000 dalton molecular weight (Aswell and Morell 1977).

This chapter also describes experiments which analysed the isolated thymocyte haemagglutinin (autorosetting receptor) and erythrocyte acceptor on SDS-PAGE. It was found that the haemagglutinin, under non-reducing conditions, had a molecular weight of 63,000 ± 2000 daltons. However, under reducing conditions the haemagglutinin gave two radioactive peaks of molecular weights 54,000 and 45,000 daltons respectively (Fig. 5.4). This result may reflect partial proteolytic cleavage of the molecule, reducing conditions releasing unlabelled fragments of molecular weight 9,000 and 18,000. Another possibility is that the haemagglutinin consists of a 45,000 dalton polypeptide associated with two subunits of 9,000 mol. wt. This point will be considered further in chapter 7. Whatever the explanation, it appears that only the unreduced haemagglutinin has binding activity as, following treatment with 2-mercaptoethanol, the haemagglutinin no longer interacted with erythrocytes as measured by uptake of radioactivity and haemagglutinating activity (data not shown).

In contrast to the haemagglutinin, the erythrocyte acceptor has a molecular weight of 62,000 daltons, both under reducing and non-reducing conditions (Fig. 5.5). This is an unusual molecular weight for H-2 antigens, even though anti-H-2L antibodies were found to block this acceptor (chapter 3). However, there is a recent report showing that a monoclonal anti-H-2 antibody (11-4.1) can immuno-precipitate a 68,000 dalton molecular weight molecule from thymoma cells (Hunter et al 1981). Interestingly, this monoclonal antibody has been shown to be carbohydrate specific (O'Neill et al 1981). Whatever the explanation for the nature of the acceptor molecule, it is a glycoprotein (chapter 4), and a carbohydrate moiety attached to this protein must be recognized by the haemagglutinin and autorosetting receptors.

Collectively the results described in this chapter imply that the H-2L/D region of the murine MHC controls a family of lectins that recognize carbohydrate structures on murine erythrocytes. Previous studies indicate that the carbohydrate acceptors on erythrocytes are also under H-2L/D region control (chapters 3 & 4), and can be blocked with anti-H-2L antibodies (chapter 3). In this context it should be noted that recent studies, using monoclonal antibodies, have defined carbohydrate H-2K^{k} antigens (O'Neill et al 1981), and these carbohydrate antigens appear to be expressed as glycolipids in cell membranes. However, in the case of the erythroctye acceptor, attachment of a carbohydrate H-2L antigen to a protein carrier may occur. On the other hand, whether the lectin is related to conventional H-2 antigens remains to be determined. Certainly the molecular weight of the lectin does not resemble the conventional 45,000 dalton protein H-2 antigens, although the molecule contains a 45,000 dalton component. Furthermore,a polypeptide of about 12,000 daltons resembling β2-microglobulin which is associated with H-2L, H-2K or H-2D antigens has so far not been detected in receptor preparations, even

when highly cross-reactive gels (12% SDS) are run. The possibility that the 54,000 dalton polypeptide may represent a complex of the β2-microglobulin associated with the 45,000 dalton protein H-2 antigen, however, can not be excluded. Preliminary studies indicate, however, that the BALB/c (H-2^d) haemagglutinin can be removed from cell lysates by a conventional anti-H-2L^d serum (AS 207-see chapter 2), but not by antisera raised against other regions of the H-2 complex (i.e., anti-H-2K^d, anti-H-2D^d and anti-Ia^d sera) (data not shown). Obviously, the ideal means of demonstrating the H-2 nature of the haemagglutinin is to assess the ability of monoclonal anti-H-2L^d and specific anti-β2-microglobulin sera to react with the purified haemagglutinin. Unfortunately, there was insufficient time before thesis submission to attempt these experiments.

Finally, it should be noted that there is one earlier report of lectins in the membranes of mouse lymphocytes (Kieda et al 1978). Whether the lectins described in this earlier study represent the same lectins discussed here is uncertain, as slightly different extraction procedures were used and a different panel of inhibitory sugars was tested.

Summary

Murine lymphocytes bind autologous erythrocytes via H-2L/H-2D region controlled receptors which appear to preferentially recognize self H-2L molecules on the erythrocyte surface. This chapter describes attempts to isolate and characterize the cell surface molecules involved in this interaction.

Initial studies revealed that detergent lysates of thymocytes contained potent haemagglutinating activity for murine erythrocytes. Several lines of experimental evidence indicated that this haemagglutinin was the autorosetting receptor, namely, (i) the haemagglutinin had a similar lymphoid organ distribution to autorosetting cells; (ii) the same

eight sugars that inhibited autorosetting also inhibited the haemagglutinin; (iii) purified autorosette inhibition factor very effectively inhibited the haemagglutinin; (iv) thymocytes from the H-2L deletion mouse strain, BALB/c-H-2^{dm2} that virtually lack autorosetting receptors, contained much less haemagglutinin than wild-type thymocytes and (v) additional genetic studies demonstrated that the haemagglutinin, like the autorosetting receptors, are controlled by the H-2L/D region of the H-2 complex.

Procedures were devised for isolating ^{125}I-labelled haemagglutinins (autorosetting receptors) and erythrocyte acceptors from detergent lysates of cells. SDS-PAGE analysis revealed that the haemagglutinin has a molecular weight of 63,000 daltons and may consist of 2-3 subunits, whereas the erythrocyte acceptor represents a single polypeptide chain of 62,000 daltons.

Chapter 6

Isolation and Biological Properties of a Serum Factor that Blocks Autorosetting Receptors on Lymphocytes

Table of Contents

6.1 Introduction

Murine thymocytes and peripheral lymphocytes can spontaneously bind autologous and allogeneic erythrocytes (Micklem and Asfi 1971, Charreire and Bach 1975, Kolb 1977 and 1978). Experiments described in chapter 3 demonstrated that thymocytes and peripheral lymphocytes bind autologous erythrocytes via receptors that primarily recognize self H-2L/H-2D molecules on the erythrocyte surface. Furthermore, on the basis of sugar-inhibition studies and the susceptibility of the autorosetting receptors and acceptors to protease and glycosidase treatments it appears that H-2L/H-2D region controlled protein receptors on thymocytes recognize the carbohydrate portion of a glycoprotein on erythrocytes (chapter 4).

Several laboratories have reported that serum can inhibit autorosette formation (Micklem and Asfi 1971, Charreire and Bach 1975, Kolb 1977). Recently Hsu et al (1980) presented evidence that the inhibitory activity (termed "autorosette inhibition factor" of AIF) was associated with the high density lipoproteins of mouse serum. In this chapter AIF is further analysed and the study demonstrates the presence of AIF in the sera of several species , its production by macrophages and its purification to homogeneity from human serum.

6.2 Results

6.2.1 Detection of Autorosette Inhibition Factor (AIF) in Mouse Serum

The ability of serial dilutions of BALB/c serum to inhibit the autorosetting of BALB/c thymocytes and spleen cells is depicted in Fig. 6.1. It can be seen that the serum completely inhibited autorosette formation at a dilution of 1/8 - 1/16 and significant inhibition was obtained with dilutions as high as 1/128. Comparable inhibition curves were obtained with both lymphoid populations even though spleen cells has a lower incidence of autorosetting cells than thymus (chapter 3).

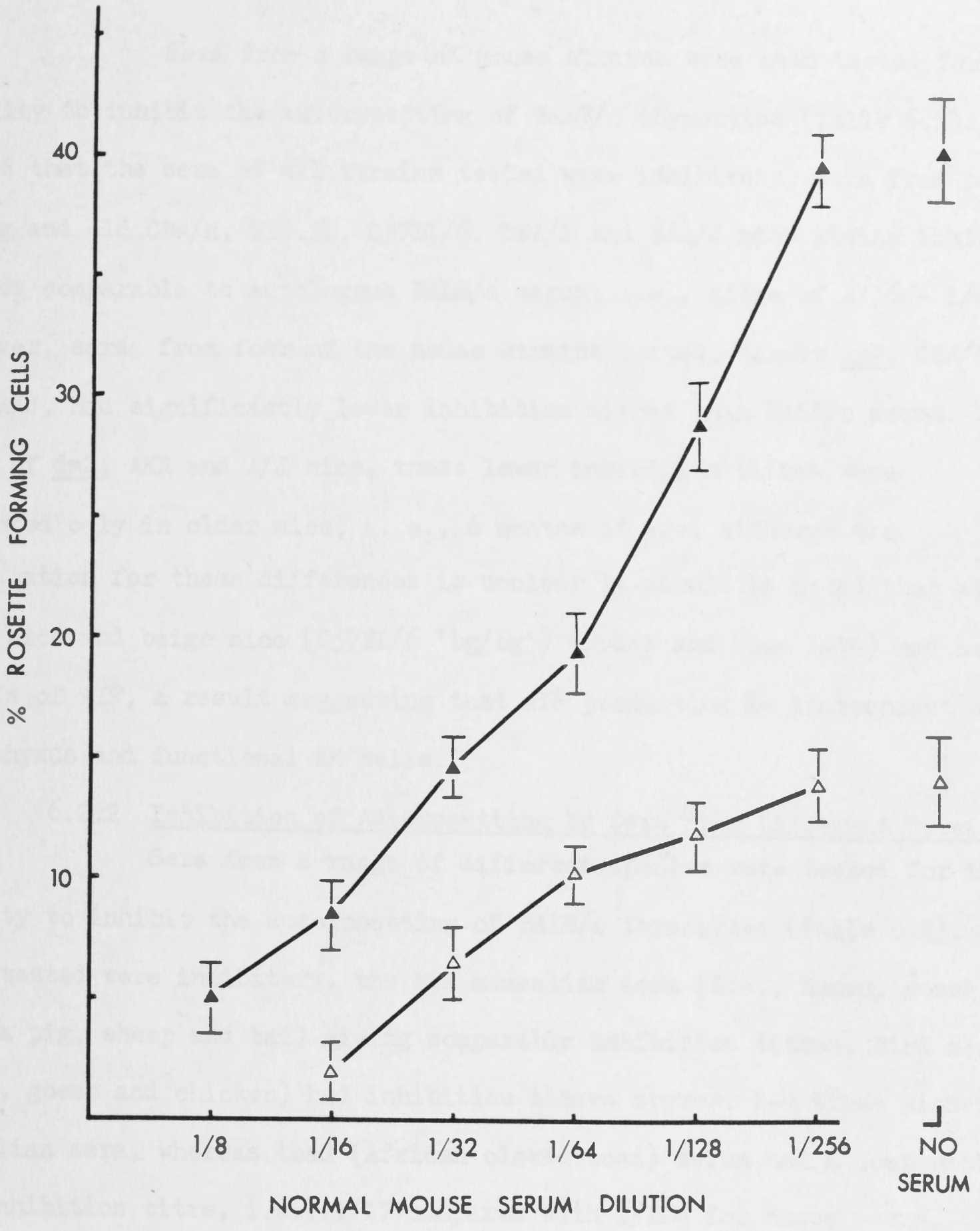

Fig. 6.1 Ability of BALB/c serum to inhibit the autorosetting of BALB/c thymocytes and spleen cells. Thymocytes (▲) or spleen cells (△) were pre-incubated with different concentrations of serum before rosetting. Vertical bars represent standard deviations of means. The rosetting of control thymocytes and spleen cells which were not preincubated with serum is indicated in the right hand margin of the figure.

Sera from a range of mouse strains were then tested for their ability to inhibit the autorosetting of BALB/c thymocytes (Table 6.1). It was found that the sera of all strains tested were inhibitory, sera from both young and old CBA/H, B10.BR, C57BL/6, DBA/1 and SJL/J mice giving inhibition titres comparable to autologous BALB/c serum, i.e., titre of 1/16 - 1/80. However, sera from four of the mouse strains tested, namely dm2, CBA/N, AKR and A/J, had significantly lower inhibition titres than BALB/c serum. In the case of dm2, AKR and A/J mice, these lower inhibition titres were detected only in older mice, i. e., 6 months of age. Although the explanation for these differences is unclear it should be noted that athymic nude mice and beige mice (C57BL/6 'bg/bg') (Roder and Duwe 1979) had normal levels of AIF, a result suggesting that AIF production is independent of the thymus and functional NK cells.

6.2.2 Inhibition of Autorosetting by Sera from Different Species

Sera from a range of different species were tested for their ability to inhibit the autorosetting of BALB/c thymocytes (Table 6.2). All sera tested were inhibitory, the six mammalian sera (i.e., human, mouse, rat, guinea pig, sheep and bat) giving comparable inhibition titres. Bird sera (i.e., goose and chicken) had inhibition titres approx. 2-4 times higher than mammalian sera, whereas toad (African clawed toad) serum had a comparatively low inhibition titre, i.e., 1/17 compared with 1/128 for mouse serum.

6.2.3 Ontogeny of AIF in Mouse Serum

Figure 6.2 depicts the levels of AIF in the sera of foetal and new born mice. The 16-day foetus had approximately 16-32 times less AIF in its serum than adult BALB/c mice. There was a gradual increase in the levels of AIF in foetal serum with age, a mouse at birth having approximately 1/4 - 1/2 the adult level. By 5-6 days after birth, adult levels of AIF in serum were detected. It is noteworthy that the ontogeny of serum AIF coincides with the ontogeny of autorosetting cells (Steele and Cunningham 1980).

Table 6.1

Ability of Serum from Different Mouse Strains to Inhibit BALB/c Autorosetting

Serum Donor	H-2 Haplotype	Age (month)	Serum Inhibitory Titre [a]
BALB/c	d	2-3	64 ± 3 [b]
		6-7	72 ± 4
BALB/c athymic nude	d	2-3	68 ± 4
BALB/c-H-2^{dm2} (C55)	dm^2	2-3	58 ± 4
		6	18 ± 4
CBA/H	k	2-3	80 ± 4
		6-7	72 ± 5
CBA/H	k	2-3	30 ± 4
		4-5	24 ± 5
B10.BR	k	2-3	78 ± 4
		6	64 ± 5
NZB	k	9	88 ± 4
AKR	k	2-3	80 ± 4
		6	16 ± 4
C57BL/6	b	2-3	85 ± 5
		6-7	70 ± 4
C57BL/6 ("+/bg")	b	2-3	68 ± 4
C57BL/6 ("bg/bg")	b	2-3	62 ± 5
DBA/1	q	2-3	60 ± 5
		5-6	74 ± 4
SJL/J	s	2-3	68 ± 4
		6-8	76 ± 5
A/J	a	2-3	67 ± 3
		5-7	22 ± 5

a) BALB/c thymocytes preincubated for 30 min at 4°C with serum from different mouse strains before rosetting with autologous erythrocytes.

b) Results are expressed as the serum dilution required to inhibit thymocyte autologous rosetting to 10% ± standard error of mean of three determinations. Values that represent inhibition titres significantly different from autologous BALB/c serum are enclosed.

Table 6.2

Ability of Serum from Different Species to Inhibit BALB/c Autorosetting

Serum Donor	Serum Inhibitory titre
Human	80 ± 4
Mouse	68 ± 5
Rat	64 ± 4
Guinea Pig	72 ± 4
Sheep	68 ± 5
Little Brown Bat	68 ± 5
Goose	256 ± 6
Chicken	160 ± 5
African Clawed Toad	17 ± 5

Footnotes as in Table 6.1

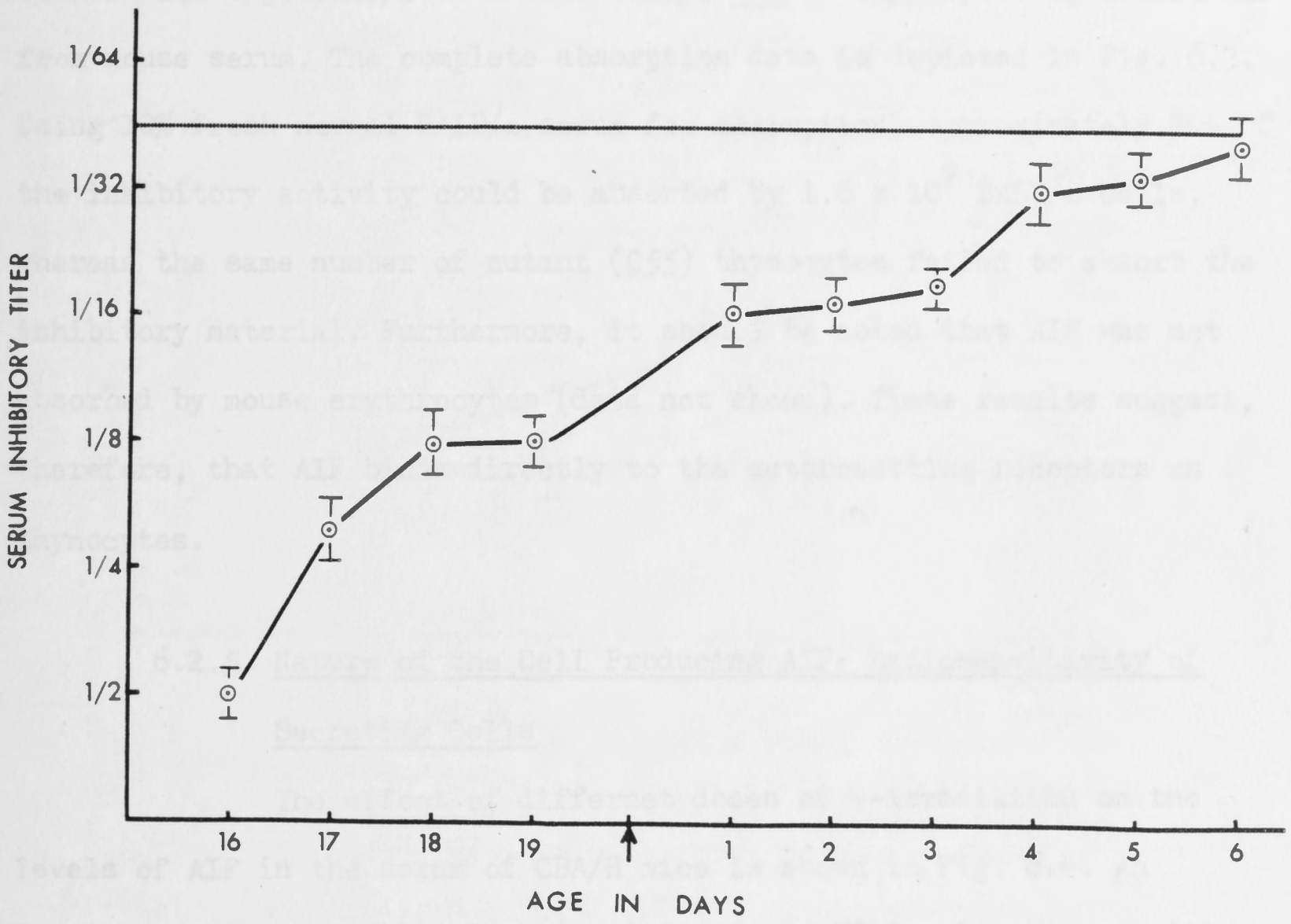

Fig. 6.2 Level of AIF in the serum of foetal and new-born BALB/c mice. The normal level of the serum inhibitory factor in adult BALB/c mice is indicated as a dotted line. Arrow denotes day of birth.

6.2.4 Absorption of AIF by Thmocytes

To establish whether AIF in serum binds directly to autorosetting receptors on thymocytes, advantage was taken of the early observation that thymocytes from the H-2L deletion mutant mouse strain, BALB/c-H-2^{dm2} (C55) lack autorosetting receptors (chapter 3). The experimental protocol was to measure the ability of varying numbers of either wild-type BALB/c or mutant BALB/c-H-2^{dm2} thymocytes to absorb AIF from mouse serum. The complete absorption data is depicted in Fig. 6.3. Using 10% fresh normal BALB/c serum for absorption, approximately 70% of the inhibitory activity could be absorbed by 1.6×10^7 BALB/c cells, whereas the same number of mutant (C55) thymocytes failed to absorb the inhibitory material. Furthermore, it should be noted that AIF was not absorbed by mouse erythrocytes (data not shown). These results suggest, therefore, that AIF binds directly to the autorosetting receptors on thymocytes.

6.2.5 Nature of the Cell Producing AIF: Radiosensitivity of Secreting Cells

The effect of differnet doses of γ-irradiation on the levels of AIF in the serum of CBA/H mice is shown in Fig. 6.4. An irradiation dose of 250 rads had no effect on AIF levels whereas with dose of 500 rads and 700 rads, there was a 50% decrease in the level of serum AIF 4-10 days after irradiation compared with untreated controls. Irradiation with 900 rads had a more marked effect on AIF production, the serum levels of AIF 6-10 days after irradiation being approximately 25% that of control animals. On the other hand, injection of 900 rad γ-irradiated mice with autologous bone marrow cells 24 hr after irradiation prevented the decline in AIF levels in serum (Fig. 6.4). These data suggest that production of AIF is dependent upon a comparatively radio-

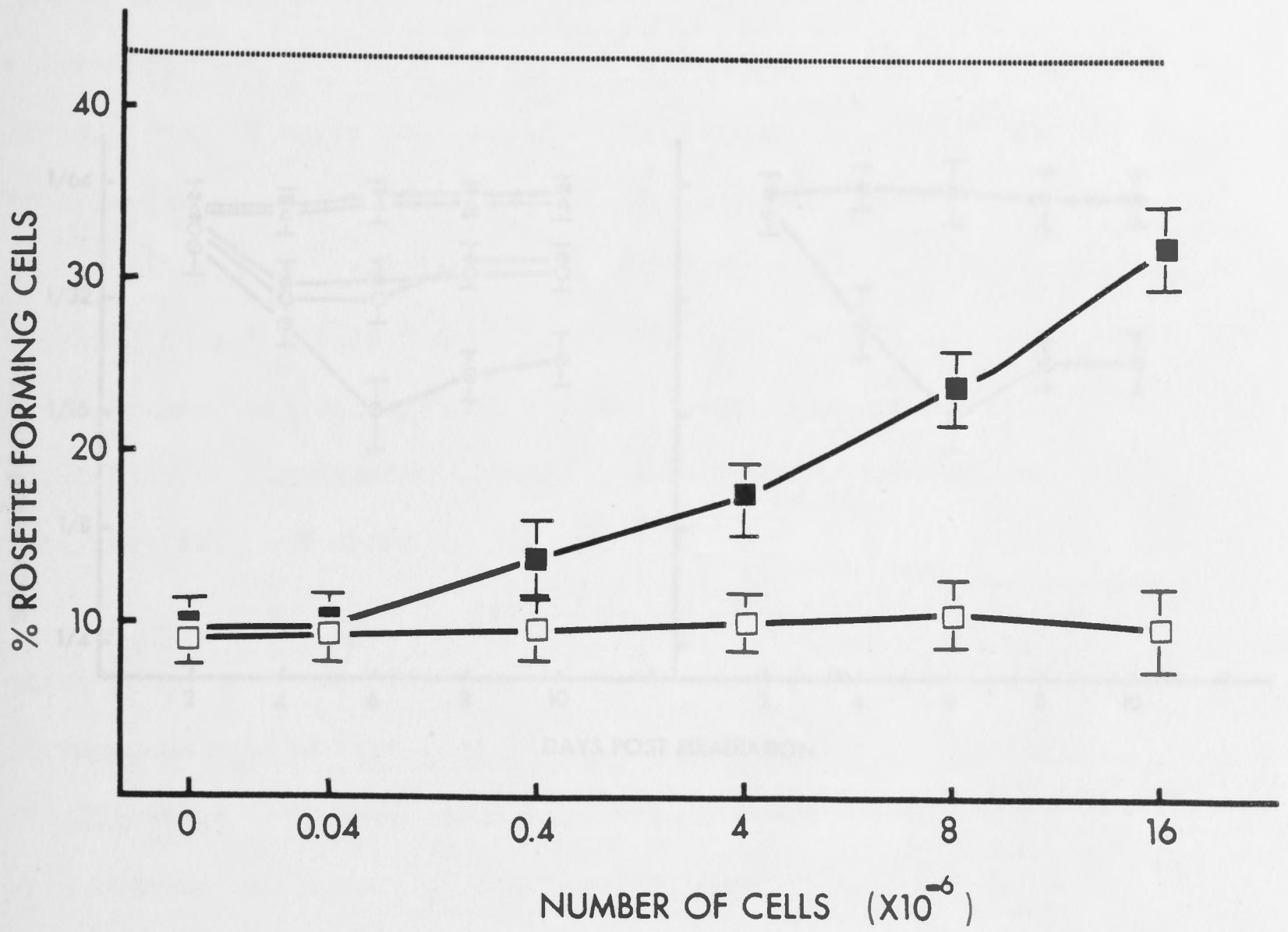

Fig. 6.3 Ability of BALB/c and BALB/c-H-2^{dm2} (C55) thymocytes to absorb AIF from BALB/c serum. 100 µl of 10% fresh BALB/c serum was preincubated with 100 µl of varying number of BALB/c or C55 thymocytes before being used to inhibit the autorosetting of BALB/c thymocytes. Vertical bars represent standard deviations of means. The rosetting of control thymocytes is shown by the dotted line.

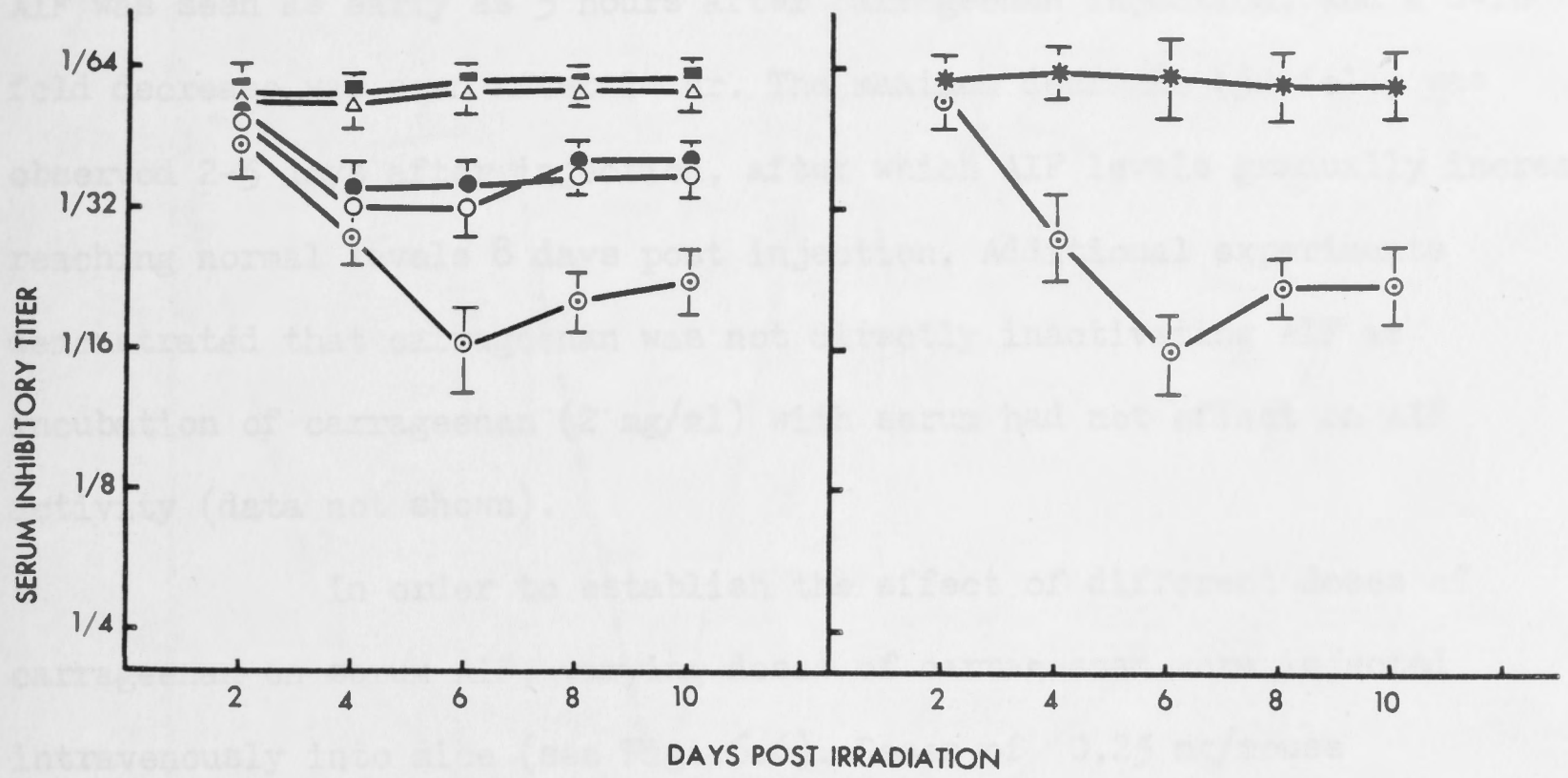

Fig. 6.4 Effect of different doses of γ-irradiation (left hand graph) and γ-irradiation followed by bone marrow reconstitution (right hand graph) on the level of AIF in the serum of CBA/H mice.

Left hand graph: Treatments were no irradiation (△), 250 (■), 500 (●), 750 (○) and 900 (⊙) rads.

Right hand graph: Treatments were 900 rad γ-irradiated mice (⊙) or mice 900 rad γ-irradiated and reconstituted with 2×10^7 bone marrow cells (✳).

sensitive cell that either resides in or is derived from bone marrow cells.

6.2.6 Sensitivity of AIF Secretion to Anti-Macrophage Agents

To determine whether serum AIF was macrophage dependent, mice were intravenously injected with anti-macrophage agents, i.e., carrageenan and silica (see Fig. 6.5). A significant decrease in the level of serum AIF was seen as early as 5 hours after carrageenan injection, and a 8-16 fold decrease was seen after 24 hr. The maximum decrease (32 fold) was observed 2-5 days after injection, after which AIF levels gradually increased, reaching normal levels 8 days post injection. Additional experiments demonstrated that carrageenan was not directly inactivating AIF as incubation of carrageenan (2 mg/ml) with serum had not effect on AIF activity (data not shown).

In order to establish the effect of different doses of carrageenan on serum AIF, varying doses of carrageenan were injected intravenously into mice (see Fig. 6.6). Doses of 0.25 mg/mouse significantly depressed serum AIF levels, there being a linear relationship between the amount of carrageenan injected and the serum AIF level.

Silica injection also depressed serum AIF levels but with very different kinetics to carrageenan (Fig. 6.5). The level of serum AIF remained unaffected for two days after silica injection, decreased 20-30 fold on day 3, but returned to normal levels by day 4. Such an effect was observed in five mice in two separate experiments.

6.2.7 Secretion of AIF by Lymphoid Cells *in vitro*

Studies reported in the preceding section with anti-macrophage agents suggest that macrophages secret AIF. As a direct test of this possibility different populations of lymphoid cells from BALB/c mice were cultured *in vitro* for 6 hr and the inhibitory activity of the

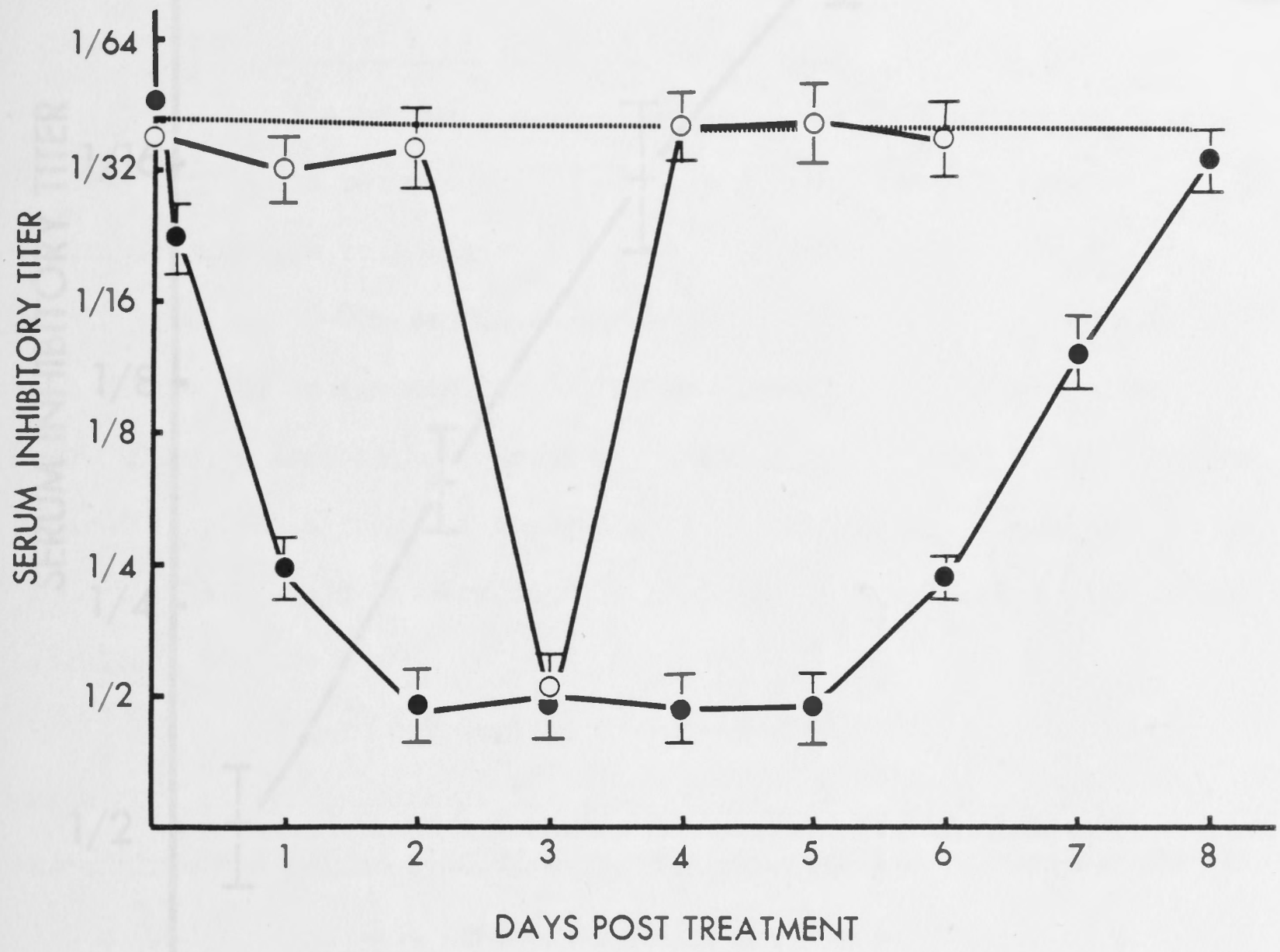

Fig. 6.5 Time course of the effect of carrageenan (●) and silica (○) injections on the level of AIF in serum. Two mg of carrageenan or silica was injected, iv, into adult CBA/H mice. The level of AIF was assessed by inhibition of auto-rosetting of CBA/H thymocytes.

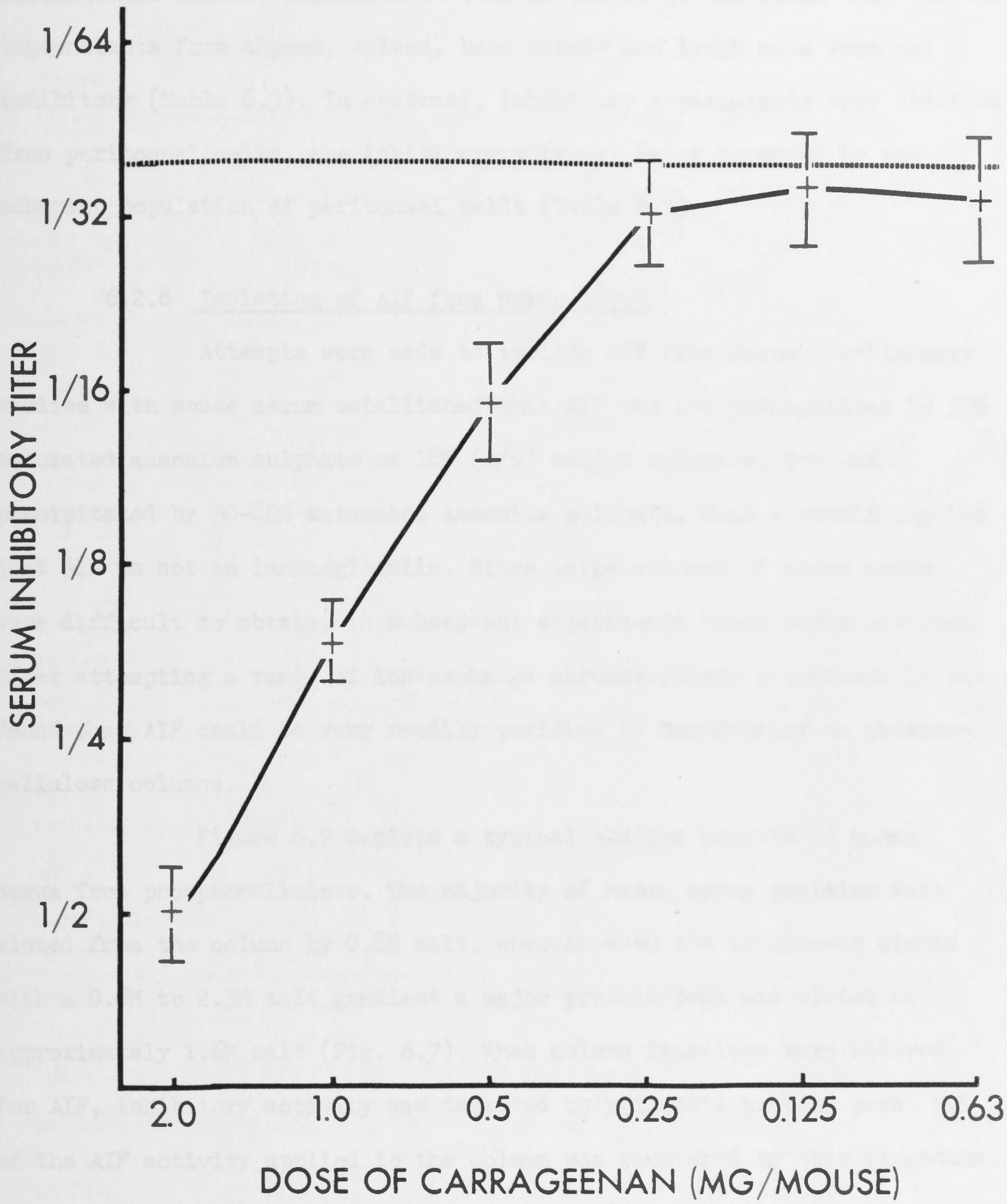

Fig. 6.6 Effect of varying doses of carrageenan on the levels of AIF in serum. Carrageenan was injected, i.v., into adult CBA/H mice, and the levels of AIF were assessed on day 3 post injection.

concentrated culture supernatants then assessed. It was found that culture supernatants from thymus, spleen, bone marrow and lymph node were not inhibitory (Table 6.3). In contrast, inhibitory supernatants were obtained from peritoneal cells, the inhibitory material being produced by the adherent population of peritoneal cells (Table 6.3).

6.2.8 Isolation of AIF from Human Serum

Attempts were made to isolate AIF from serum. Preliminary studies with mouse serum established that AIF was not precipitated by 50% saturated ammonium sulphate or 18% (w/v) sodium sulphate, but was precipitated by 50-80% saturated ammonium sulphate. Such a result implies that AIF is not an immunoglobulin. Since large volumes of mouse serum were difficult to obtain, in subsequent experiments human serum was used. After attempting a range of ion-exchange chromatography procedures it was found that AIF could be very readily purified to homogeneity on phospho-cellulose columns.

Figure 6.7 depicts a typical elution profile of human serum from phosphocellulose. The majority of human serum proteins were eluted from the column by 0.8M salt, whereas when the column was eluted with a 0.8M to 2.5M salt gradient a major protein peak was eluted at approximately 1.6M salt (Fig. 6.7). When column fractions were assayed for AIF, inhibitory activity was detected only in this protein peak. 72% of the AIF activity applied to the column was recovered by this procedure.

Fractions 50-70 were pooled and concentrated by vacuum dialysis. Analysis of the pooled fraction by SDS-polyacrylamide gel electrophoresis revealed that the preparations contained only a single Coomassie Blue staining protein band of molecular weight 81,000 daltons (Fig. 6.8).

Table 6.3

Inhibitory Activity of Cell Culture Supernatants

Source of supernatant [a]	No. of cells cultured	Volume (ml)	% autorosetting cells [b]
Nil	-	-	44 ± 4.5 [c]
Thymus	10^8	5	40 ± 6.1
Lymph Node	10^8	5	39 ± 4.7
Spleen	10^8	5	39 ± 4.6
Bone marrow	10^8	5	37 ± 4.9
Peritoneal cells	2×10^7	2	7 ± 5.2
Non-adherent peritoneal cells	10^7	1	39 ± 5.5
Adherent peritoneal cells	10^7	1	10 ± 3.7

a) Supernatants harvested and concentratred 15 fold from BALB/c cells cultured for 6 hr at 37°C in F15 medium.

b) BALB/c thymocytes preincubated with undiluted supernatant for 30 min at 4°C before rosetting with autologous erythrocytes.

c) Results expressed as % autorosetting thymocytes ± standard error of three determinations. Values that represent significant inhibition are enclosed in brackets.

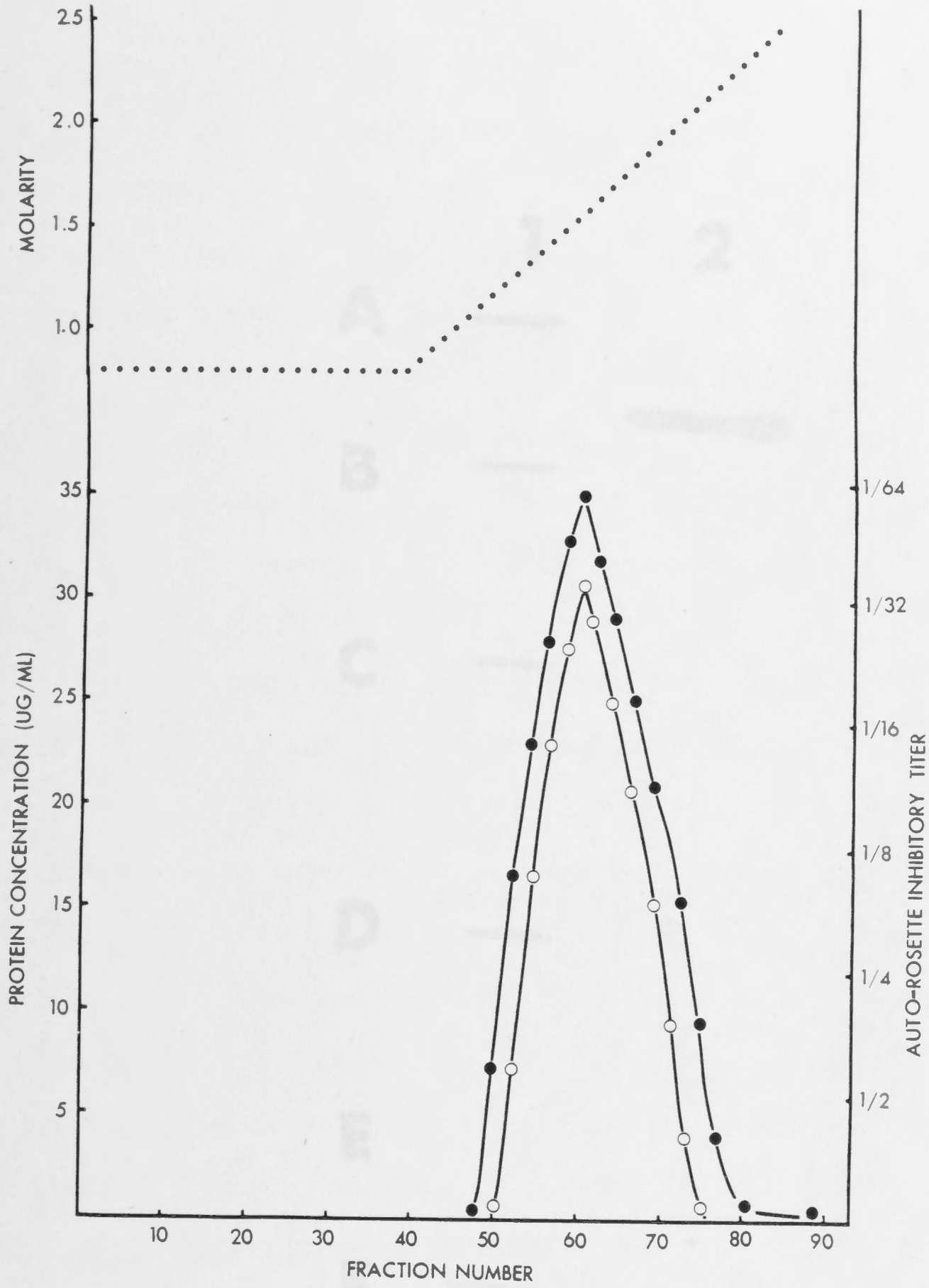

Fig. 6.7 Isolation of AIF by ion exchange chromatography on a phosphocellulose column. Human plasma in citrate buffer was applied to a column (3 X 20 cm) which had been precycled with BSA and then equilibrated with 0.8M NaCl, containing 1mM EDTA; 1mM DTT and 10 mM sodium phosphate, PH 7.4 (as described in Materials and Methods). The column was then eluted with a linear NaCl gradient from 0.8M to 2.5M (see upper graph). Fractions of 5 ml were collected and tested for protein concentration (●) and AIF activity (○).

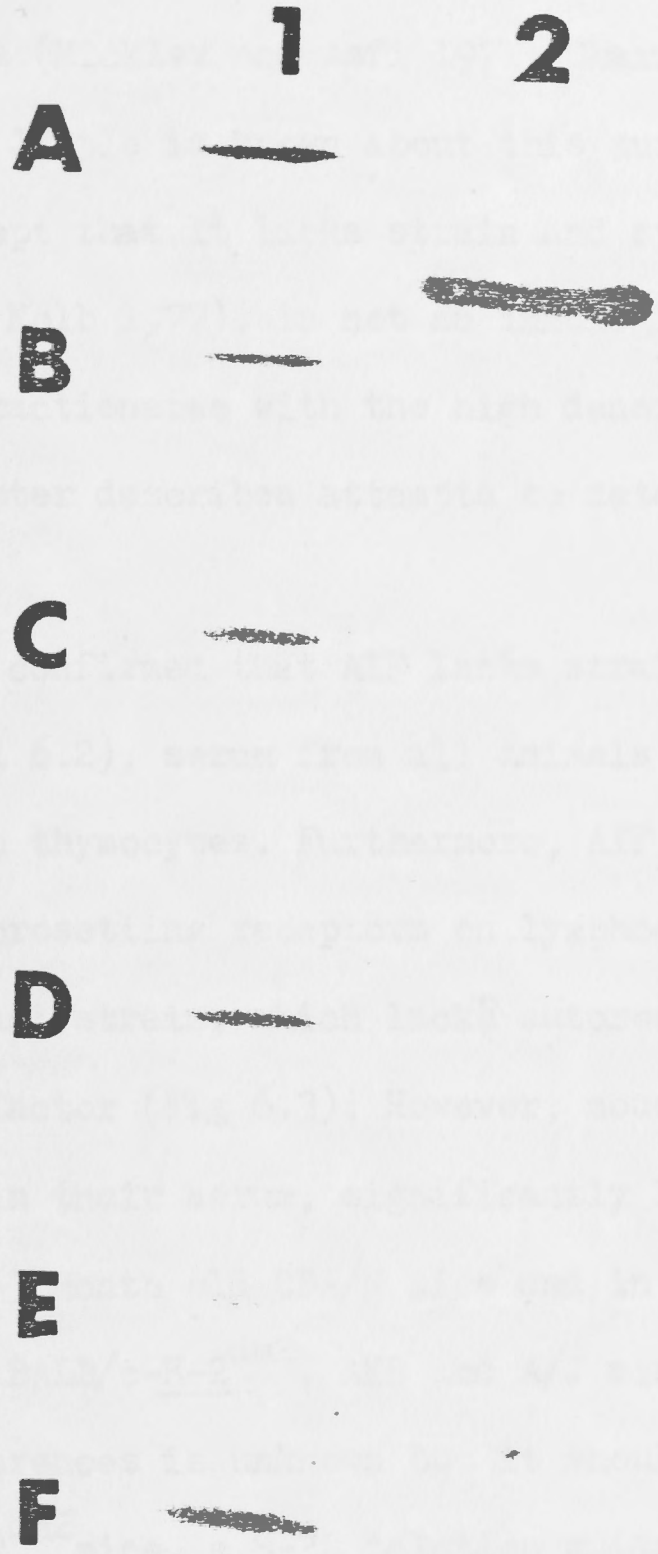

Fig.6.8. SDS-polyacrylamide gel electrophoresis of concentrated phosphocellulose column fractions nos. 50-75 from elution profile depicted in Fig.6.7: (1) Molecular weight standards: A: Phosphorylase B (97,400) B: BSA (68,000) C: ovalbumin (43,000) D: Carbonic anhydrase (30,000) E: soybean trypsin inhibitor (21,000) F: lysozyme (14,000) (2) AIF

6.3 Discussion

In the past ten years a number of laboratories have reported that normal serum can effectively inhibit rosetting between lymphocytes and autologous erythrocytes (Micklem and Asfi 1971, Charreire and Bach 1975, Kolb 1977). However, little is known about this autorosette inhibition factor (AIF) in serum, except that it lacks strain and species specificity (Charririre and Bach 1975, Kolb 1977), is not an immunoglobulin (Kolb 1977) and in mouse serum fractionates with the high density lipoproteins (Hsu et al 1980). This chapter describes attempts to determine the origin and nature of serum AIF.

Initial studies confirmed that AIF lacks strain and species specificity (Tables 6.1 and 6.2), serum from all animals tested inhibiting the autorosetting of BALB/c thymocytes. Furthermore, AIF appears to directly interact with autorosetting receptors on lymphocytes as thymocytes from the BALB/c-H-2^{dm2} mutant strain, which lacks autorosetting receptors, were unable to absorb the factor (Fig 6.3). However, mouse strains did vary in the levels of AIF in their serum, significantly lower inhibition titres being detected in 2-3 month old CBA/N mice and in old (6 months) but not young (2-3 months) BALB/c-H-2^{dm2}, AKR and A/J mice. The explanation for these differences is unknown but it should be noted that lymphocytes from BALB/c-H-2^{dm2} mice, a H-2L deletion mutant (Morgan et al 1978), almost completely lack autorosetting receptors (chapter 3). It appears unlikely that the H-2L region directly controls AIF synthesis as lymphocytes from both 2 month-old and 6 month-old mice lack autorosetting receptors whereas AIF levels are depressed only in older mutant mice. It is conceivable, however, that expression of autorosetting receptors could indirectly effect AIF production. On the other hand, there must be another explanation for the lower levels of serum AIF in CBA/N, AKR and A/J mice as these strains contain normal proportions of autorosetting cells in their lymphoid organs (data not shown).

An additional, interesting feature of these experiments was that serum from old NZB mice, a strain that spontaneously develops a range of autoimmune diseases (Staats 1980), expressed normal levels of AIF. This result suggests that development of autoimmunity in this strain is not due to a lack of AIF, although low levels of AIF may be the cause of autoimmune disorders in other strains. However, analysis of human serum from patients with a range of autoimmune diseases (i.e., rheumatoid arthritis, systemic lupus erythematosis, thyrotoxicosis and myasthenia gravis) revealed no change in serum levels of AIF (data not shown).

In this study a wider range of species was tested for serum AIF than previously reported. It is interesting to note that not only all mammalian sera, but bird (goose and chicken) and amphibia (toad) sera contained inhibitory activity. It could be argued that bird and amphibia sera contain "non-specific" inhibitory factors unrelated to mammalian AIF. However, the inhibitory molecule has been isolated from chicken serum and has similar properties to human AIF (D. B. Rylatt, personal communication).

Several experimental approaches established the nature of the AIF secreting cells. First, the demonstration of normal levels of AIF in the serum of athymic nude mice and NK cell defective beige mice (Table 6.1) suggested that AIF secretion is independent of T lymphocytes and NK cells. Second, total body irradiation experiments suggested that AIF production is dependent upon a comparatively radio-sensitive cell that is bone marrow derived (Fig. 6.4). Third, <u>in vivo</u> secretion of AIF was highly sensitive to the anti-macrophage agents, i.e., carrageenan and silica 6.5 & 6.6). Fourth, <u>in vitro</u> culturing of different lymphoid cells revealed that AIF was secreted by an adherent population of peritoneal cells (Table 6.3). Collectively these data suggest that AIF is secreted by a population of short-lived, radiosensitive macrophages (or monocytes).

An intriguing aspect of these experiments was that although

the two anti-macrophage agents carrageenan and silica profoundly depressed serum AIF levels they differed markedly in their kinetics of action (Fig. 6.5). Carrageenan lowered AIF levels rapidly and for a prolonged period (days 1-7 after injection) whereas silica only affected the serum AIF titre on the third day after injection. The explanation for this difference is uncertain but presumably reflects the target cell and mode of action of these anti-macrophage agents. For example, silica may delete a precursor of the AIF secreting cells (hence the delay in action) whereas carrageenan may directly inactivate the AIF producing cells. Whatever the explanation of these findings, the sudden fluctuations in AIF levels observed in these experiments suggest that there is a rapid turnover of AIF in serum.

This chapter also described a single step procedure using phosphocellulose ion-exchange chromatography for isolation of AIF from human serum (Fig. 6.7). Precycling of the column with BSA and thorough washing of the column after absorption of human serum are essential steps in the isolation of human AIF. Using this procedure pure AIF is obtained as judged by SDS-polyacrylamide gel electrophoresis (Fig. 6.8). The inhibitory protein has a molecular weight of 81,000 daltons when electrophoresed under reducing conditions. Furthermore, gel chromatography on Sephacryl S200 revealed a single species of molecular weight 79,000 daltons, thus implying the active protein is a single polypeptide chain (D. B. Rylatt personal communication). AIF appears to be present in serum at a concentration of the order of 50-100 µg/ml of human serum. Further characterization of this protein and the analysis of its interaction with the lymphocyte receptor may lead to a role being assigned to this serum factor <u>in vivo</u>.

Summary

Murine lymphocytes spontaneously bind autologous and allogeneic erythrocytes via receptors that primarily recognize self-H-2L/H-2D molecules on the erythrocyte surface. Normal mouse serum contains a factor, termed autorosette inhibition factor, (AIF), that very effectively blocks autorosette formation. This report describes experiments that determine the origin and nature of serum AIF.

It was found that AIF lacks strain and species specificity, serum from several mammalian and non-mammalian species inhibiting the autorosetting of BALB/c thymocytes. However, mouse strains differed in the levels of AIF in their serum. Furthermore, AIF appears to directly interact with autorosetting receptors on lymphocytes as thymocytes from the BALB/c-H-2^{dm2} mutant strain, which lack autorosetting receptors, were unable to absorb the factor.

Several lines of experimental evidence indicated that AIF is secreted by a population of short-lived, radiosensitive macrophages (or monocytes). First, *in vivo* administration of the anti-macrophage agents, i.e., carrageenan and silica profoundly depressed AIF levels in serum. Second, *in vitro* culturing of different lymphoid cells revealed that AIF is secreted by an adherent population of peritoneal cells. Third, total body irradiation experiments demonstrated that AIF production is dependent upon radiosensitive cells that are bone marrow derived.

Finally, a single step procedure was devised for purifying AIF to homogeneity from human serum. Purified AIF was found to be a single polypeptide chain with a molecular weight of 81,000 daltons.

Chapter 7

General Discussion

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7.1 Introduction

Substantial subpopulations of murine lymphocytes can form rosettes with both autologous and allogeneic erythrocytes (Micklem and Asfi 1971, Sandilands *et al* 1974, Charriere and Bach 1975, Braganza *et al* 1975, Kolb 1977). Although this observation was first reported ten years ago there is still little known about the phenomenon. The studies described in this thesis attempted to correct this deficiency by analysing the autorosetting phenomenon at four levels:

(1) The involvement of MHC gene products in the interaction

(2) The molecular basis of the interaction

(3) The manner in which autorosetting receptors are regulated *in vivo*.

(4) The possible functional significance of the autorosetting receptors *in vivo*

This final discussion will summarise my experimental findings and discuss their overall biological significance.

Involvement of H-2 Complex in Autorosetting

There is increasing evidence that the MHC in mice (H-2 complex) and other species is involved in various immune phenomena (reviewed in chapter 1). Thus, self H-2K and H-2L/D antigens are involved in the recognition of foreign antigens by cytotoxic T cells (Doherty *et al* 1976, Shearer 1976) whereas I-region controlled antigens are recognized in association with foreign antigen by the T lymphocytes that express help (Katz and Benacerraf 1975), delayed-type hypersensitivity (Miller *et al* 1976), and antigen specific proliferation *in vitro* (Thomas *et al* 1977, Schwartz *et al* 1978). Based on these observations it appeared possible that autorosetting may be mediated by an anti-self receptor that recognizes a MHC gene product on erythrocytes.

The experimental data presented in chapter 3 indicates that autorosetting is mediated by H-2L/H-2D region controlled receptors on thymocytes as well as peripheral T and B lymphocytes that preferentially recognize self H-2L/H-2D region controlled molecules on the erythrocyte surface. This conclusion was based on three lines of evidence. First, by measuring the ability of erythrocyte sonicates from different mouse strains to inhibit rosette formation it was found that only sonicates from H-2L/H-2D region compatible strains were inhibitory. This result implied that the autorosetting receptors had highest affinity for self H-2L/H-2D region controlled structures on the erythrocytes. Second, anti-H-2L sera were able to specifically block inhibition of autorosetting by erythrocyte sonicates, a result suggesting recognition of H-2L antigens was involved. Third, it was found that H-2L/H-2D mutant strains of mice (B10.D2-H-2^{dm1} and BALB/c-H-2^{dm2}) simultaneously expressed altered autorosetting receptors and acceptors, suggesting that the H-2L/H-2D region controls autorosetting at both the thymocyte and erythrocyte level.

At the same time that these investigations were being carried out two reports appeared in the literature which also demonstrated that autorosetting was under MHC control, i.e., lymphocytes preferentially bound H-2 compatible erythrocytes (Primi et al 1979, Charriere et al 1980). However, in these two separate studies autorosetting was not mapped to the H-2L/H-2D region. By rosetting 24 hr cultured spleen cells with a variety of H-2 congenic and recombinant strains of murine erythrocytes, Primi et al (1979) detected preferential binding of H-2 compatible erythrocytes and mapped autorosetting to an undefined H-2 locus, between H-2G and H-2L/H-2D. This is a surprising finding because uncultured spleen cells and thymocytes are found to rosette equally well with autologous, allogeneic and xenogeneic erythrocytes (Micklem and Asfi 1971, Sandilands et al 1974, Charriere and Bach 1975, Braganza et al

1975, Kolb 1977). One possible explanation for these differences is that cultured spleen cells recognize different structures on erythrocytes than uncultured splenocytes, i.e., culturing selects for a subpopulation of autorosetting cells. Another important point is that Primi *et al* a much lower proportion of autorosetting cells than in the studies presented in this thesis. This is probably due to the workers employing suboptimal autorosetting conditions (discussed in chapter 2). Again this could result in a selected subpopulation of autorosetting cells being examined.

In the second study Charriere *et al* (1980) used two approaches to assess *H-2* involvement in autorosetting. First, like Primi *et al* (1979), they observed preferential binding of *H-2* compatible erythrocytes. In the second approach they assessed the ability of erythrocyte ghosts from different mouse strains to block autorosetting. Based on the data of one *H-2* recombinant mouse strain B10.A ($\underline{K}^k$, $\underline{D}^d$), they concluded that both *K* and D regions were involved in autorosetting. However, this study only examined one recombinant between *k* and *d* haplotypes, and the data in chapter 3 demonstrated that the autorosetting receptors of *k* and *d* haplotypes are completely cross reactive, i.e., genetic mapping studies cannot be attempted between these haplotypes.

Once autorosetting was shown to be *H-2L*/*H-2D* region controlled it was important to determine whether the specificity of the autorosetting receptors, like the specificity of *H-2* restricted T cells (Zinkernagel and Doherty 1979), was influenced by the thymic environment. By analysing lymphocytes from allogeneic and semi-allogeneic chimeras (chapter 3, section 3.2.10), it was found that the radioresistant thymic epithelium of the chimeric mice did not modify the specificity of the autorosetting receptors on the donor lymphocytes. This result implied that the autorosetting receptors are

controlled in a different manner to the functional anti-self receptors on cytotoxic T lymphocytes, a point which will be further discussed in section 7.6 of this chapter.

7.3 Nature of the Autorosetting Receptors and Acceptors

Once autorosetting had been shown to be under H-2L/H-2D region control, it was important to determine the chemical nature of the autorosetting receptors and acceptors. Initial studies described in chapter 3 (section 3.2.8) indicate that although the majority (80%) of autorosetting spleen cells are B lymphocytes, the autorosetting receptors are not immunoglobulin or immunoglobulin-associated. This finding confirms similar experiments by Primi et al (1979) but is at variance with work by two other groups (Micklem and Asfi 1971, Steele and Cunningham 1980). However, the anti-Ig reagents used by these two groups were not immunoabsorbent purified and would have contained AIF, a potent blocker of autorosetting (chapter 6).

Chapters 4 and 5 are devoted to characterizing the autorosetting receptors and acceptors. Several lines of evidence indicate that autorosetting is mediated by a protein receptor on lymphocytes (thymocytes) that interacts with a carbohydrate acceptor on erythrocytes. Briefly, this data is as follows:

(1) A range of simple sugars could inhibit autorosetting in a strain specific manner, these strain differences mapping to the H-2L/D region of the MHC (section 4.2.1).

(2) The erythrocyte acceptor was destroyed by certain exoglycosidases whereas the thymocyte receptor was glycosidase resistant. Furthermore, difference in glycosidase susceptibility of the acceptor was noted between mouse strains, an effect again mapped to the H-2L/D region (section 4.2.2).

(3) Both the thymocyte receptor and erythrocyte acceptor were protease sensitive, a finding which suggested that the carbohydrate on erythrocytes was protein bound (section 4.2.2).

(4) The autorosetting receptor could be detected in detergent lysates of thymocytes and was characterized as a H-2L/H-2D region controlled lectin (chapter 5).

A combination of the data obtained suggested that the autorosetting receptors on thymocytes recognize terminal D-galactose, D-mannose and sialic acid residues on a branched chain carbohydrate structure on erythrocytes, mouse strains of different H-2 haplotype expressing carbohydrate structures that differ in the linkage of these three terminal sugars.

Based on the above findings procedures were devised for isolating the autorosetting receptors and acceptors from ^{125}I-labelled detergent lysates of thymocytes and erythrocytes, respectively. SDS-PAGE analysis revealed that the haemagglutinin has a molecular weight of 63,000 daltons and under reducing conditions gives fragments of molecular weight 45,000 and 54,000 daltons.

Three possible structures of the autorosetting receptor derived from these observations are depicted in the figure shown on the next page. It could be that the autorosetting receptor is a polypeptide chain of 63,000 daltons consisting of a 45,000 dalton polypeptide chain disulphide bonded to two subunits, each of molecular weight 9,000 daltons (Fig. A). If reduction was incomplete, components of 45,000 and 54,000 daltons would appear. The second possibility is that the autorosetting receptor contains two proteolytic cleavage sites, each separated by a 9,000 dalton unit (Fig. B). As a result of proteolysis, one or two of these cleavage sites may be affected, thus

Fig. A

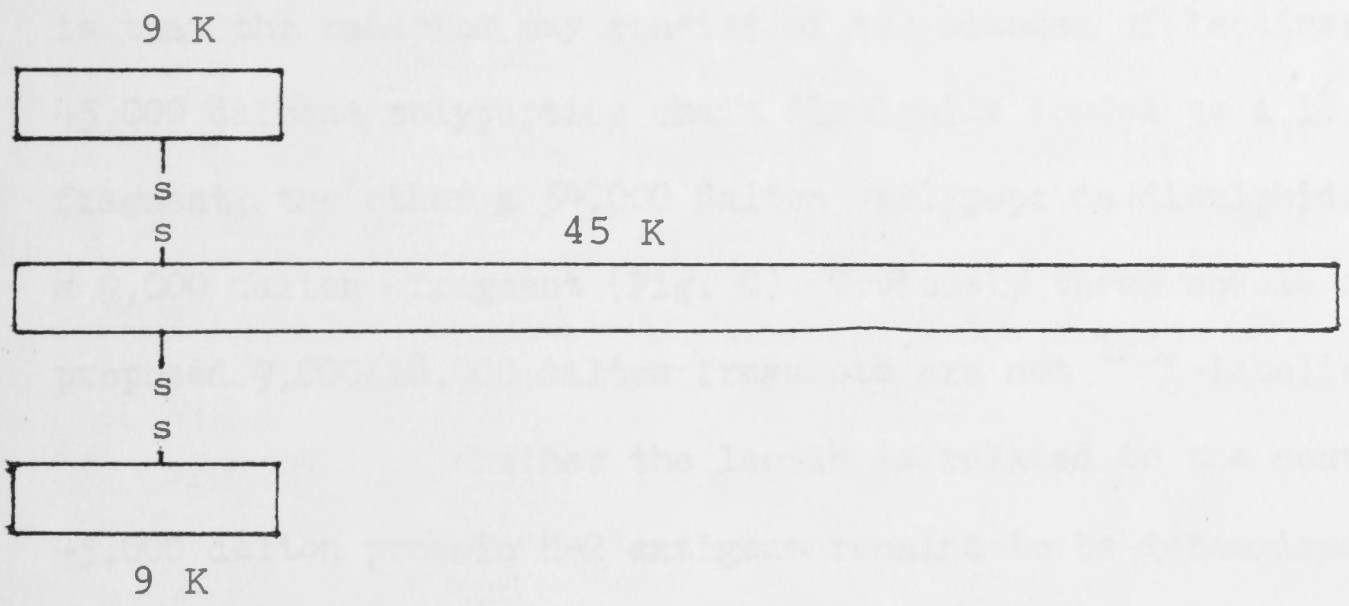


Fig. B

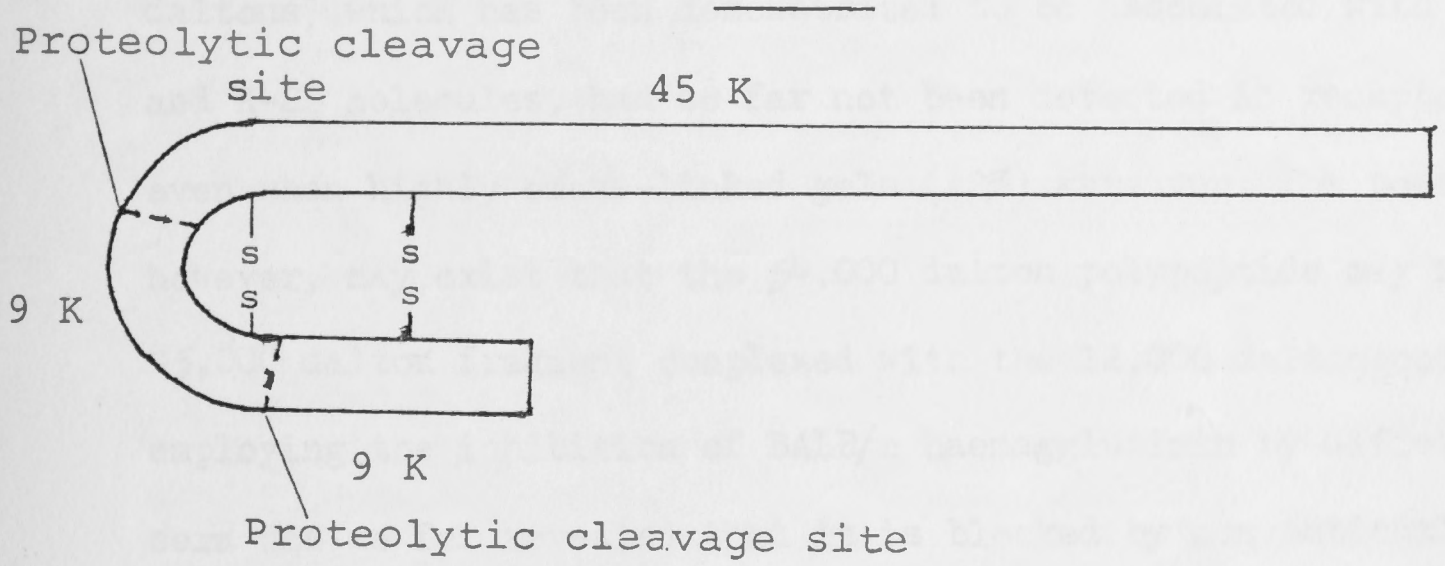
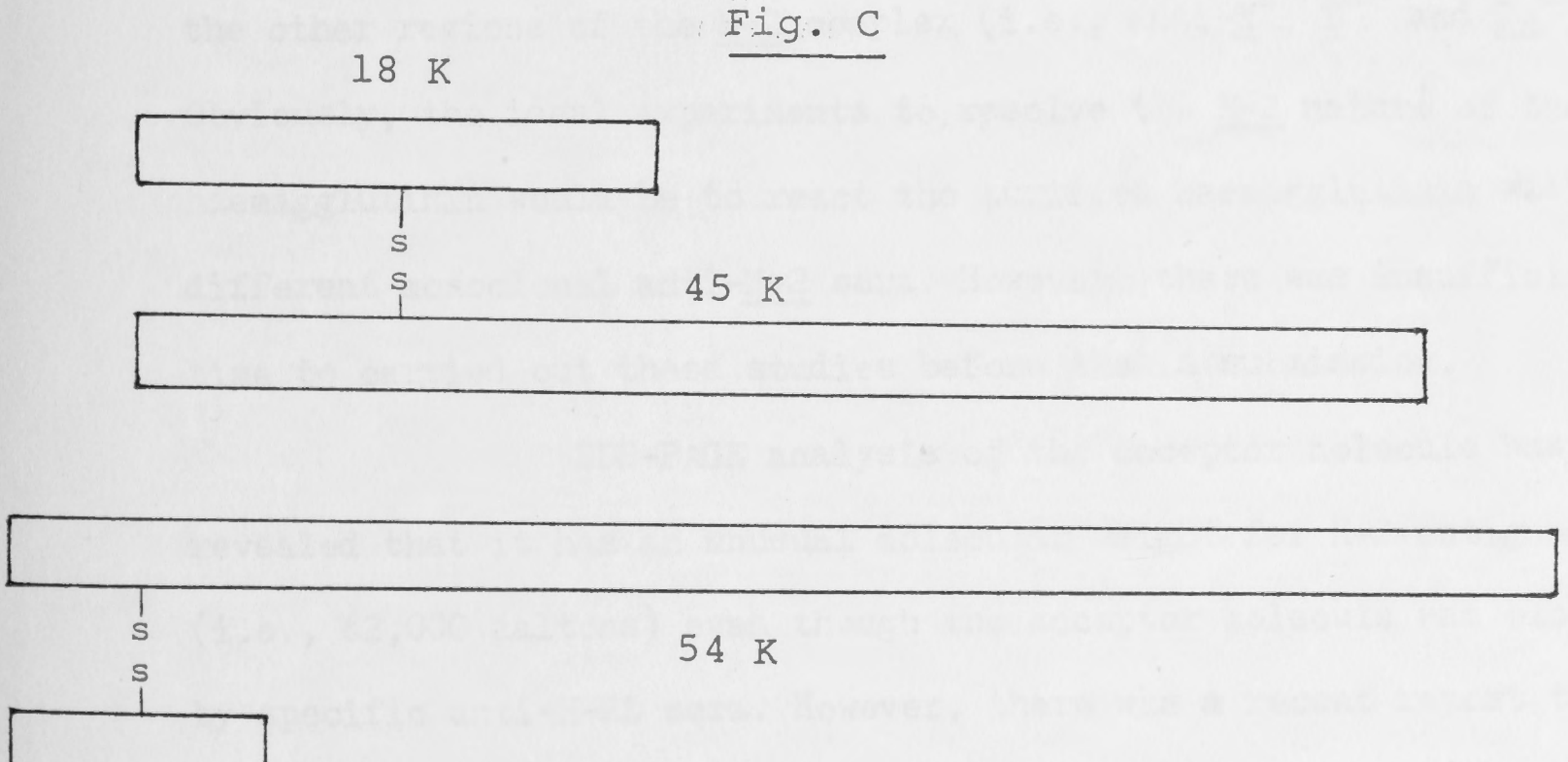


Fig. C



Possible Structures of the Autorosetting Receptor (Haemagglutinin)

releasing either 45,000 or 54,000 dalton fragments. The third possibility is that the receptor may consist of two classes of lectins: one of a 45,000 daltons polypeptide chain disulphide bonded to a 18,000 dalton fragment; the other a 54,000 dalton polypeptide disulphide bonded to a 9,000 dalton fragment (Fig. C). Obviously these models assume the proposed 9,000/18,000 dalton fragments are not ^{125}I-labelled.

Whether the lectin is related to the conventional 45,000 dalton protein H-2 antigens remains to be determined. A polypeptide similar in molecular size to β2-microglobulin (i.e., 12,000 daltons),which has been demonstrated to be associated with H-2K, H-2D and H-2L molecules, has so far not been detected in receptor preparations even when highly cross-linked gels (12%) were run. The possibility , however, may exist that the 54,000 dalton polypeptide may represent the 45,000 dalton fragment complexed with the 12,000 dalton component. Studies employing the inhibition of BALB/c haemagglutinin by different anti-H-2 sera has so far revealed that it is blocked by conventional anti-H-2L^d antibodies (AS 207) (data not shown), but not antibodies raised against the other regions of the H-2 complex (i.e., anti-K^d, D^d, and Ia^d). Obviously, the ideal experiments to resolve the H-2 nature of the haemagglutinin would be to react the purified haemagglutinin with different monoclonal anti-H-2 sera. However, there was insufficient time to carried out these studies before thesis submission.

SDS-PAGE analysis of the acceptor molecule has also revealed that it has an unusual molecular weight for H-2 antigens (i.e., 62,000 daltons) even though the acceptor molecule was blocked by specific anti-H-2L sera. However, there was a recent report that a monoclonal anti-H-2 antibody (11-4.1) could immuno-precipitate a 68,000 dalton molecular weight molecule from thymoma cells (Hunter et al, 1981).

Interestingly, this monoclonal antibody has been shown to be carbohydrate specific (O'Neill et al 1981). Whatever the relationship between the acceptor molecule and H-2 it appears to be a glycoprotein (chapter 4), the carbohydrate moiety attached to it being recognized by autorosetting receptors.

7.4 Specificity of the Autorosetting Phenomenon

At this point certain aspects of the specificity of the autorosetting receptors should be emphasized. First, it appears that autorosetting lymphocytes bind syngeneic, allogeneic and xenogeneic (rat) erythrocytes by the same receptor (chapter 3). However, studies with erythrocyte sonicates revealed that the autorosetting receptor has highest affinity for H-2L/H-2D region compatible structures. In chapter 3 (section 3.3) it was suggested that sonicates detected this "self preference" because (i) binding of intact red cells represents multi-point binding whereas the membrane vesicles generated by sonication would not bind in such a multi-point fashsion and (ii) unbound sonicates were washed away from lymphocytes whereas in rosetting assays unbound red cells were not removed. In this context, the ability of Primi et al (1979) and Charriere et al (1980) to detect preferential binding of intact red cells may be due to the very vigorous resuspension of rosettes by these workers (i.e., vigorous mixing on a mechanical roller for 30 - 60 sec), which would favour highest affinity receptors. Perhaps a good demonstration that sonicate-inhibition highlights self preference was the observation that dm2 mutant erythrocytes (H-2L deletion), although rosetting normally with thymocytes of all mouse strains when intact, were unable to inhibit wild-type ($H\text{-}2^d$) autorosetting when used as a sonicate. This observation also indicates that the dm2 mutant has defective autorosetting receptors and acceptors.

Second, if one accepts that the autorosetting receptors have highest affinity for self H-2L/H-2D molecules but cross react with other H-2 antigens, why is it that the detergent solubilised autorosetting receptors (haemagglutinin) described in chapter 5 fail to show haplotype specificity in their haemagglutinating activity? The possible explanation for this paradox is that, following detergent depletion by XAD-8 beads the autorosetting receptors exist as high molecular weight aggregates rather than as separate molecules. Certainly gel filtration studies indicate that the haemagglutinin in these preparations has a molecular weight of approximately 500,000 daltons (D.B. Ryaltt, personal communication). Thus the haemagglutinin would bind to the red cells in a multi-point fashion and consequently, similar to autorosetting, react with allogeneic and xenogeneic (rat) red cells. Whether the solubilised erythrocyte acceptor also exists as a high mol. wt. complex in detergent-depleted lysates has not been determined. The report by Aswell and Morell (1977) that the galactose-binding lectin of hepatocytes consists of two subunits of mol. wt. 48,000 and 40,000 daltons that form high mol. wt. complexes of 500,000 daltons is consistent with mammalian lectins forming high mol. wt. aggregates.

Finally, sonicate-inhibition studies revealed that F1 lymphocytes simultaneously express receptors against both parents (chapter 3, section 3.2.9). This conclusion was based on the observation that erythrocyte sonicates of either parent were potent inhibitors of F1 lymphocyte rosetting. Thus, this finding would seem to be a paradox as if there were separate receptors against each parental H-2 haplotype one would only expect autorosette inhibition when erythrocyte sonicates from both parents were present. However, it was suggested in chapter 3 (section 3.3.3) that an explanation for this

paradox is that the erythrocyte sonicates represent membrane vesicles (i.e., the vesicles that inhibit autorosetting are sedimented by 5000 - 20,000 g centrifugation) and not solubilized membrane component. Thus, once lymphocyte receptors against one parental haplotype bound erythrocyte vesicles the receptors directed against the other parent would be sterically masked.

7.5 Regulation of Autorosetting Receptors In Vivo

Obviously the function of autorosetting receptors is not to interact with red cells in vivo. Autorosetting merely represents a convenient assay for detecting this family of anti-self receptors.

In support of this notion is the finding that serum contains a potent blocker of autorosetting receptor (termed autorosette inhibition factor or AIF). As described in chapter 6, human AIF was purified to homogeneity and shown to be a single polypeptide of mol. wt. 81,000 daltons and at a concentration of 50-100 µg/ml of human serum. An interesting feature of AIF is that it is active across species barriers, murine autorosetting being blocked by AIF from many species, e.g., human, chicken and toad. This implies that AIF plays an important function in vivo and there are strong evolutionary pressures to conserve its structure. Certainly the blocking of lymphocyte receptors that potentially, can react against most self cells is an important function. Presumably autorosetting receptors are only functional in tissues where AIF is absent (see next section).

Dr. D. B. Rylatt in this department recently has further characterized AIF from several species. He has found that human, mouse and bovine AIF's have mol. wt. of 81,000 daltons and

almost identical amino acid compositions. In contrast, chicken AIF has a mol. wt of 160,000 daltons but its amino acid composition resembles mammalian AIF. An interesting feature of the AIF's is that they are rich in histidine and proline (approx. 15% of protein) and, in fact, resemble a protein of unknown function, termed histidine rich glycoprotein, isolated from human serum several years ago (Heimburger et al 1972). Finally, it is intriguing to note that AIF is secreted by a population (populations?) of macrophages (chapter 6). As macrophages are well documented to play an important role in regulating immune responses (Escobar and Friedman 1980), the network whereby AIF regulates autorosetting receptors remains an exciting field of research.

7.6 Functional Significance of Autorosetting Receptors

As mentioned above, the autorosetting receptors on lymphocytes certainly do not function in vivo as receptors for self red cells. Also, it is unlikely that the autorosetting receptors represent the postulated "anti-self receptors" involved in H-2 restricted recognition by T lymphocytes (Zinkernagel and Doherty 1979). This conclusion is based on the following observations:

(1) The autorosetting receptors are restricted to the H-2L/H-2D region whereas cytotoxic T cells recognize self H-2K, H-2L and H-2D structures.

(2) In the periphery B lymphocytes are the predominant autorosetting cells. In fact, most data suggest that immature, rather than mature T cells, carry autorosetting receptors (Charreire and Bach 1975, chapter 3).

(3) The specificity of the autorosetting receptors is unchanged in allogeneic and semi-allogeneic chimeras, i.e., the H-2

specificity is the same as the donor cells (chapter 3). In contrast, the H-2 specificity of cytotoxic T cells is changed in these chimeras (Zinkernagel and Doherty 1979).

(4) Addition of purified human AIF to cultures at concentrations that block all autorosetting receptors failed to block a range of in vitro immune responses, such as generation and effector phases of H-2 restricted virus-specific cytotoxic T cells, primary and secondary antibody responses, generation of virus specific delayed-type hypersensitivity and mitogen responses (data not shown).

A likely function of the autorosetting receptors is that they mediate a form of intercellular adhesion in which these MHC-controlled receptors recognize MHC-controlled structures on another cell. At this point it should be noted that there are several reports in the literature of MHC-controlled intercellular adhesions. The first description of this phenomenon was by Barlett and Edidin (1978) who observed that the rate of adhesion of mouse embryo fibroblasts to fibroblast monolayers was related to the H-2 haplotype of the fibroblast monolayer. That H-2 antigens were involved in this interaction was supported by the ability of anti-H-2 sera to inhibit fibroblast-fibroblast intercellular adhesion.

Intercellular adhesion between mouse bone marrow and lymph node cells has been shown to be under H-2 control, H-2 compatible cells adhering more effectively than allogeneic cells (Zeleny et al 1978). Similarly, K and D-region compatibility between T and B cells was reported to be required for the secretion of certain factors that diminish the adhesiveness of the opposite cell type (Curtis 1978, 1979). Compatibility at the K and D regions of the H-2 complex was also shown to be necessary for controlling contact inhibition of movement of mouse epithelium outgrowths. Contact inhibition was greatly increased when

confronting epithelial outgrowths were histo-incompatible at these genetic regions (Curtis and Rooney 1979). In a comletely different system H-2 antisera were shown to be capable of blocking the interaction between murine hepatocytes and metastasizing tumour cells (Schirrmacher et al 1980). An interesting conclusion from this study was that this interaction is probably mediated by a galactose-specific lectin on the hepatocytes that results in liver metastasis.

In a recent report, it was also shown that the optimal homing of T and B lymphocytes to lymph nodes requires identity at either the K and/or D-region of the murine MHC. Thus it is likely that lymphocytes interact with endothelial cells via receptors that preferentially recognize self MHC antigens (Pincott and Bainbridge 1980).

Finally, the in vivo erythropoietic performance of mouse bone marrow cells has been demonstrated to be much lower in H-2 disparate than in H-2 identical irradiated host (Lengerova et al 1977). Studies of several H-2 recombinants and a H-2 mutant indicated that the K and D-regions of the H-2 complex were involved. In addition, it was suggested that the recognition by the host of a carbohydrate H-2 molecule on the donor cells determined erythropoietic performance.

Whether the autorosetting receptors mediate and AIF inhibits any of these other forms of intercellular adhesion is an interesting question that deserves investigation. As mention above in section 7.5, a speculative function of the autorosetting receptors is that, in the in vivo absence of AIF, they may act as a lymphocyte "homing" device. Conversely, union of the receptor with its acceptor could provide a differentiation signal. In this context, Dr. D. B. Rylatt has observed recently that AIF is highly sensitive to protease attack (particularly by plasmin) and, therefore, the local activation or release of proteases

(such as in the case of tissue damage) could result in AIF destruction, exposure of the autorosetting receptors and consequent localisation of lymphocytes at that site.

In conclusion, it should be emphasized that whatever the function of the autorosetting receptors may be, the autorosetting phenomenon appears to be under MHC control. This finding suggests that MHC not only controls the interaction of antigen-specific T lymphocytes with target cells but may play a much more fundamental role in cell-cell communication.

7.7 Implications of Autorosetting being a MHC-Controlled Protein-Carbohydrate Interaction

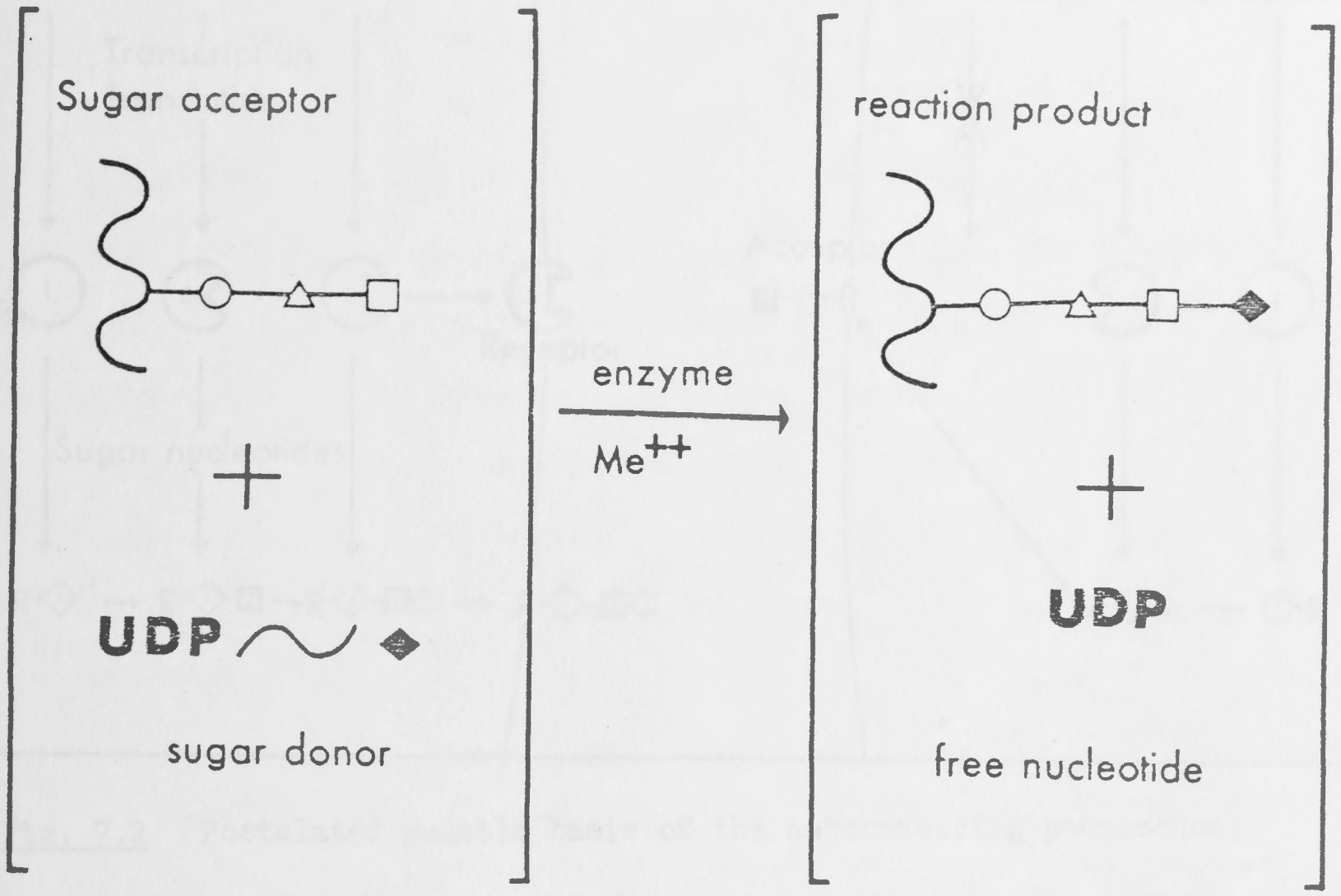
In this final section, a speculative hypothesis will be proposed which considers the theoretical implications of autorosetting being mediated by a H-2L/H-2D controlled lectin on lymphocytes that recognizes a H-2L/H-2D region controlled carbohydrate structures on erythrocytes. An obvious implication is that the H-2L/H-2D region in some way codes for carbohydrate structures. This is not the first evidence for carbohyrate structures being controlled by the murine MHC. Previous studies indicate that the I-region controls carbohydrate Ia antigens (McKenzie et al 1977, Higgins et al 1980) and recent studies with monoclonal antibodies suggest that carbohydrate H-$2K^k$ antigens exist (O'Neill et al 1981). Furthermore, these studies indicate that the carbohydrate H-2 and Ia antigens are usually expressed as glycolipids on the cell surface and are different molecules to the conventional protein H-2 and Ia antigens.

The existence of carbohydrate H-2L/H-2D structures suggests that the H-2L/H-2D region codes for the glycosyltransferase enzymes that construct these molecules. The manner in which glycosyl-

transferases catalyse the transfer of a monosaccharide residue from a sugar nucleotide (sugar donor) to the non-reducing terminus of a specific sugar acceptor is depicted in Fig. 7.1. It should be emphasized that the attachment of each sugar in a carbohydrate chain requires a separate glycosyltransferase that has specficity for both the sugar acceptor and the sugar donor. Although the simplest model is that the H-2L/H-2D region directly codes for glycosyltransferases a more complex possibility is that the glycosyltransferases are coded for by genes located outside the MHC and the H-2L/H-2D region produces regulators of these enzymes.

Once one considers glycosyltransferases as a gene product it is not difficult to envisage how one genetic region can control the expression of two separate classes of molecules on the cell surface. Thus the primary gene product of the H-2L/H-2D would be a family of glycosyltransferases that construct the carbohydrate H-2L/H-2D antigens, and both of these molecules could be expressed in the cell membrane as separate entities. Furthermore, it can be seen from Fig. 7.1 that glycosyltransferases basically are lectins and, therefore, the autorosetting receptors on lymphocytes could be H-2L/H-2D controlled glycosyltransferases. Such a model, in which glycosyltransferases on the surface of one cell recognize their carbohydrate substrate on another cell, has been proposed by Roseman (1970) to explain specific cell-cell communication. Fig. 7.2 depicts the way in which this model can be adapted to the autorosetting phenomenon, where a H-2L/D region controlled glycosyltransferase (transferase 3) is expressed on the surface of lymphocytes and interacts with its substrate, an incompletely glycosylated H-2L/D carbohydrate, on erythrocytes.

Is it possible to explain the modified autorosetting behaviour of H-2L/H-2D mutant mice by this model? In the case of the H-2L deletion mutant ($H\text{-}2^{dm2}$), where there is a simultaneous loss of the



GLYCOSYLTRANSFERASE REACTION

Fig.7.1 Glycosyltransferase reaction. Various monosaccharides are represented by circles, triangles, squares and diamonds. A trisaccharide sugar acceptor is depicted, linked at its reducing end to a protein or lipid moiety represented by the wavy line. The enzyme, usually in the presence of divalent metal ions, catalyzes the transfer of a monosaccharide from its uridine diphosphate donor to the nonreducing terminus of the sugar acceptor. The reaction products are a tetrasaccharide, represented here with a terminal "diamond" moiety, and the free nucleotide. From Shur and Roth (1975).

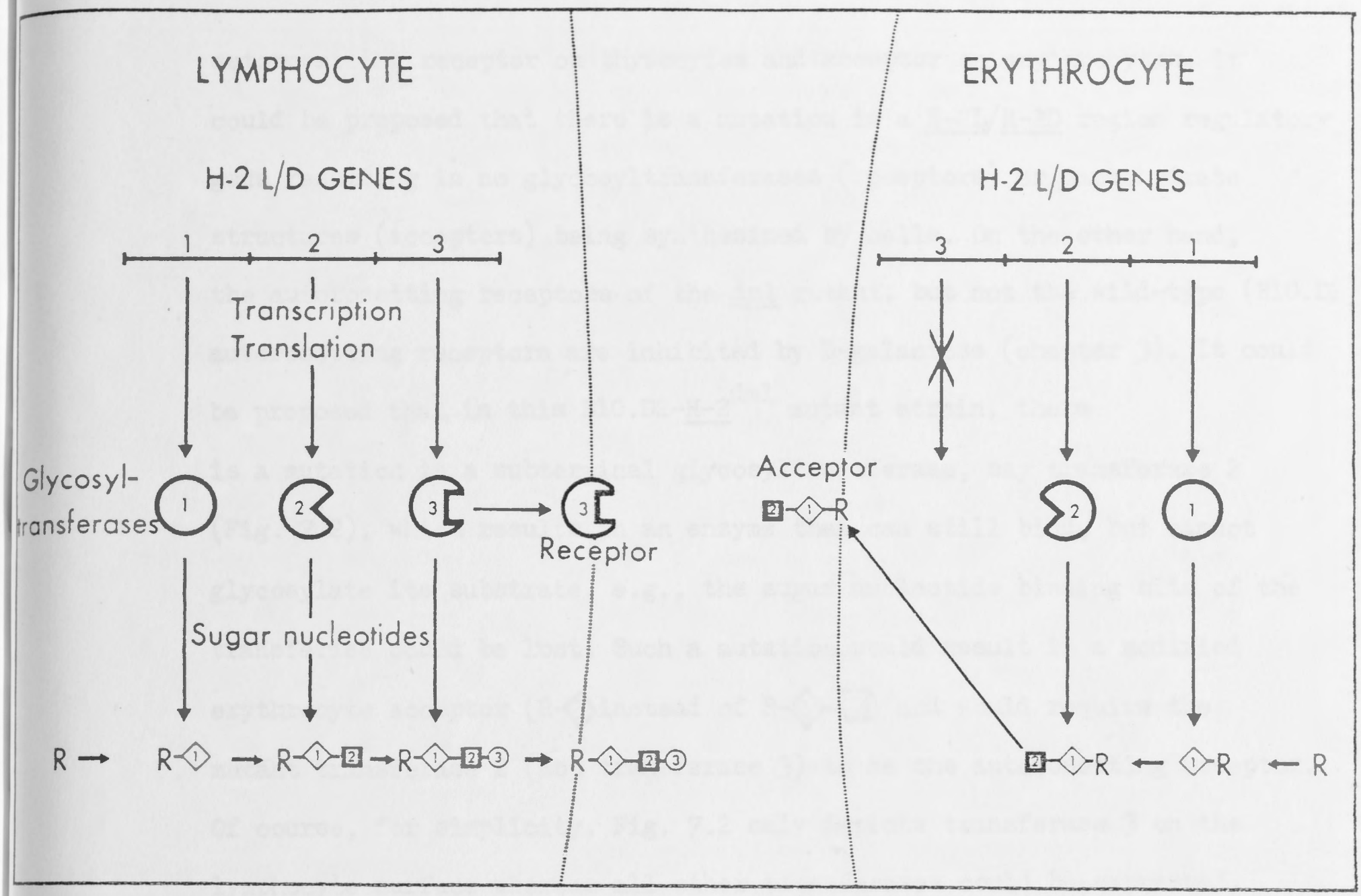


Fig. 7.2 Postulated genetic basis of the autorosetting phenomenon.

The H-2L/D region is depicted as coding for three different glycosyltransferases that sequentially attach three different sugars (depicted as diamonds, squares and circles) to a core structure (R). Thus glycosyltransferase 1 catalyses the attachment to R of sugar ◇1 from its sugar nucleotide precursor, transferase 2 catalyses the attachment of sugar □2 to sugar ◇1, etc. It is proposed that glycosyltransferase 3 is expressed on the surface of lymphocytes and acts as the autorosetting receptor. In contrast, transferase 3 is not produced by erythrocytes and, therefore, an incomplete carbohydrate structure (R—◇1—□2) is expressed on the surface of erythrocytes and serves as the autorosetting acceptor. The model is derived from Roseman's generalised hypothesis for cell-cell recognition.

autorosetting receptor on thymocytes and acceptor on erythrocytes, it could be proposed that there is a mutation in a H-2L/H-2D region regulatory gene resulting in no glycosyltransferases (receptors) or carbohydrate structures (acceptors) being synthesized by cells. On the other hand, the autorosetting receptors of the dml mutant, but not the wild-type (B10.D2) autorosetting receptors are inhibited by D-galactose (chapter 3). It could be proposed that in this B10.D2-H-2^{dml} mutant strain, there is a mutation in a subterminal glycosyltransferase, say transferase 2 (Fig. 7.2), which results in an enzyme that can still bind, but cannot glycosylate its substrate, e.g., the sugar nucleotide binding site of the transferase could be lost. Such a mutation would result in a modified erythrocyte acceptor (R-◇1 instead of R-◇1-□2) and would require the mutant transferase 2 (not transferase 3) to be the autorosetting receptor. Of course, for simplicity, Fig. 7.2 only depicts transferase 3 on the lymphocyte surface whereas all other transferases could be expressed.

An important question arising from this model is whether conventional H-2 protein antigens are, indeed, glycosyltransferases. The 63,000 dalton molecular weight of the autorosetting receptor (chapter 5) does not, however, resemble the conventional 45,000 dalton protein H-2 antigens, although the receptor appears to contain a 45,000 dalton component. Obviously, this question can only be answered by determining whether different anti-H-2 sera, particularly monoclonal anti-H-2L antibodies, react with the purified autorosetting receptors.

Another important question arising from these studies is whether H-2 restricted cytotoxic T cells (Zinkernagel and Doherty 1979) interact with target cells in a manner similar to autorosetting cells. Are the target H-2 antigens recognized by cytotoxic T cells carbohydrate and are the postulated anti-self receptors on these cells lectins? Currently there is no experimental evidence to discount these

questions but it should be possible to devise experiments to answer them.

Appendix:

Assaying Autorostting Thymocytes By The Coulter Counter

A microscope method has been used by different research groups to detect autorosetting lymphocytes in the mouse and other species. Obviously, this microscope assay is subjective, tedious and time consuming. For these reasons, an automated procedure for counting autorosettes was devised using Coulter Counter, a machine which can determine the number and size of particles suspended in a electrically conductive liquid. The Coulter Counter achieves this by forcing the suspension to flow through a small aperture having an immersed electrode on either side. As a particle passes through the aperture, it changes the resistance between the electrodes. This produces a voltage pulse of short duration having a magnitude proportional to the particle size. The series of pulses is then electronically scaled and counted by the machine. In the case of autorosetting it would be anticipated that autorosettes would be larger than free red cells, and non-rosetting lymphocytes and, therefore, could be readily counted by this machine.

In the experiments presented single cell suspensions of BALB/c thymocytes and erythrocytes were prepared as described in chapter 2. Thymocytes were made up to 4×10^6/ml and erythrocytes to 0.75% in HBSS containing 0.5% FCS. For calibrating the machine, 200 µl of the thymocyte suspension was mixed with 200 µl of the 0.75% erythrocyte suspension. The mixture was centrifuged at 200 g for 1 min at 4°C. The cell pellet was allowed to incubate on ice for 1 hr before being resuspended gently using a short pasteur pipette. For Coulter Counter counting, the mixture was then gently pipetted into 20 ml of cold PBS and mixed gently. The counting profiles obtained for erythrocytes alone, autorosetting thymocytes, and autorosetting thymocytes in the presence of 10%

autologous serum (sufficient serum to block autorosettes) at various threshold settings are depicted in the lower graph of Fig. 1. It can be seen that autorosetting thymocytes gave a different size distribution to non-rosetting (serum blocked) thymocytes, increasing lower threshold values representing increases in cell volume. The optimal number of autorosetting thymocytes (23%) was obtained at a lower threshold setting of 36 (upper graph of Fig. 1).

A parallel microscope assay detected 45% autorosetting cells in the sample. This discrepancy may be due to disruption of same autorosettes as they passed through the orifice. This is unlikely, however, as the machine also gives lower values for highly stable antibody-mediated rosettes. The discrepancy is probably due to autorosetting thymocytes having a wide size distribution, hence no one threshold setting can detect all autorosettes.

In subsequent experiments a lower threshold setting of 40 was chosen as this setting detected a high proportion of autorosettes but few free thymocytes. For serum inhibition of autorosetting, 200 µl of the thymocyte suspension (4×10^6/ml) was incubated with 500 µl of neat, or serial two fold dilutions of BALB/c serum for 30 min on ice. 200 µl of a 0.75% autologous erythrocyte suspension was then added for rosette formation. The percent autorosette forming cells obtained at each serum dilution revealed that the inhibition profile of autorosetting by the different serum dilutions was comparable to that obtained with the microscope assay (Fig. 2). This probably reflects differences in the minimum size of autorosettes detected in the two assays.

The results obtained in this study show that the Coulter Counter assay can replace the microscope method as a non-subjective method for quantitating autorosetting lymphocytes.

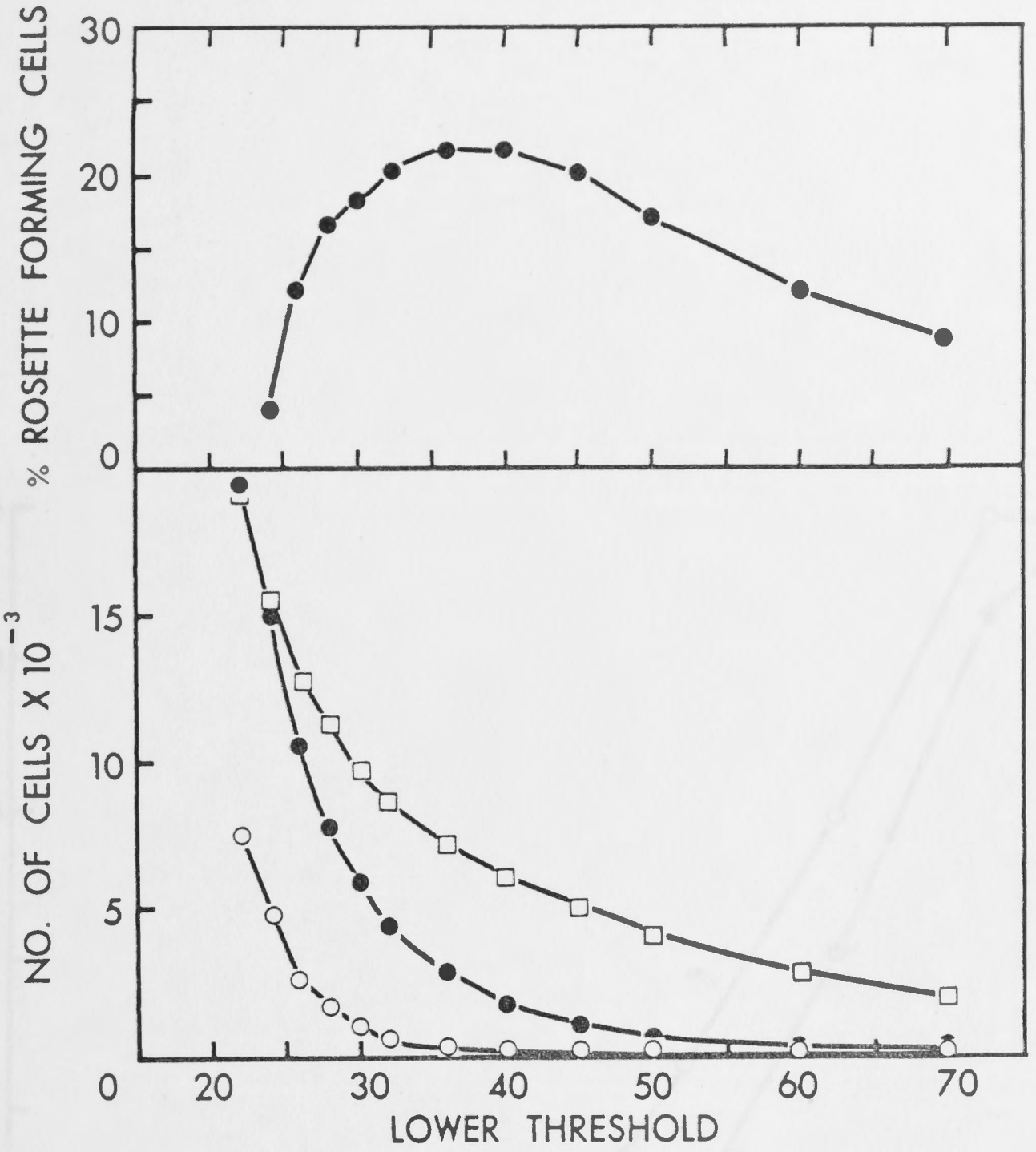


Fig. 1. Detection of autorosetting thymocytes by the Coulter Counter. The lower graph depicts the number of cells counted at each lower threshold setting, the curves depicting BALB/c mouse red cells alone (○), BALB/c thymocytes + BALB/c red cells rosetted in presence of 20% BALB/c serum (●) and BALB/c thymocytes + BALB/c red cells rosetted in the absence of mouse serum (□). The upper graph depicts the percent rosette forming cells calculated at each lower threshold setting according to the formula:

$$\text{\% RFC at lower threshold setting X (LTX)} = \frac{\text{No. cells in curve (□) at LTX} - \text{No. cells in curve (●) at LTX}}{\text{Total no. thymocytes in sample}}$$

For further details see text.

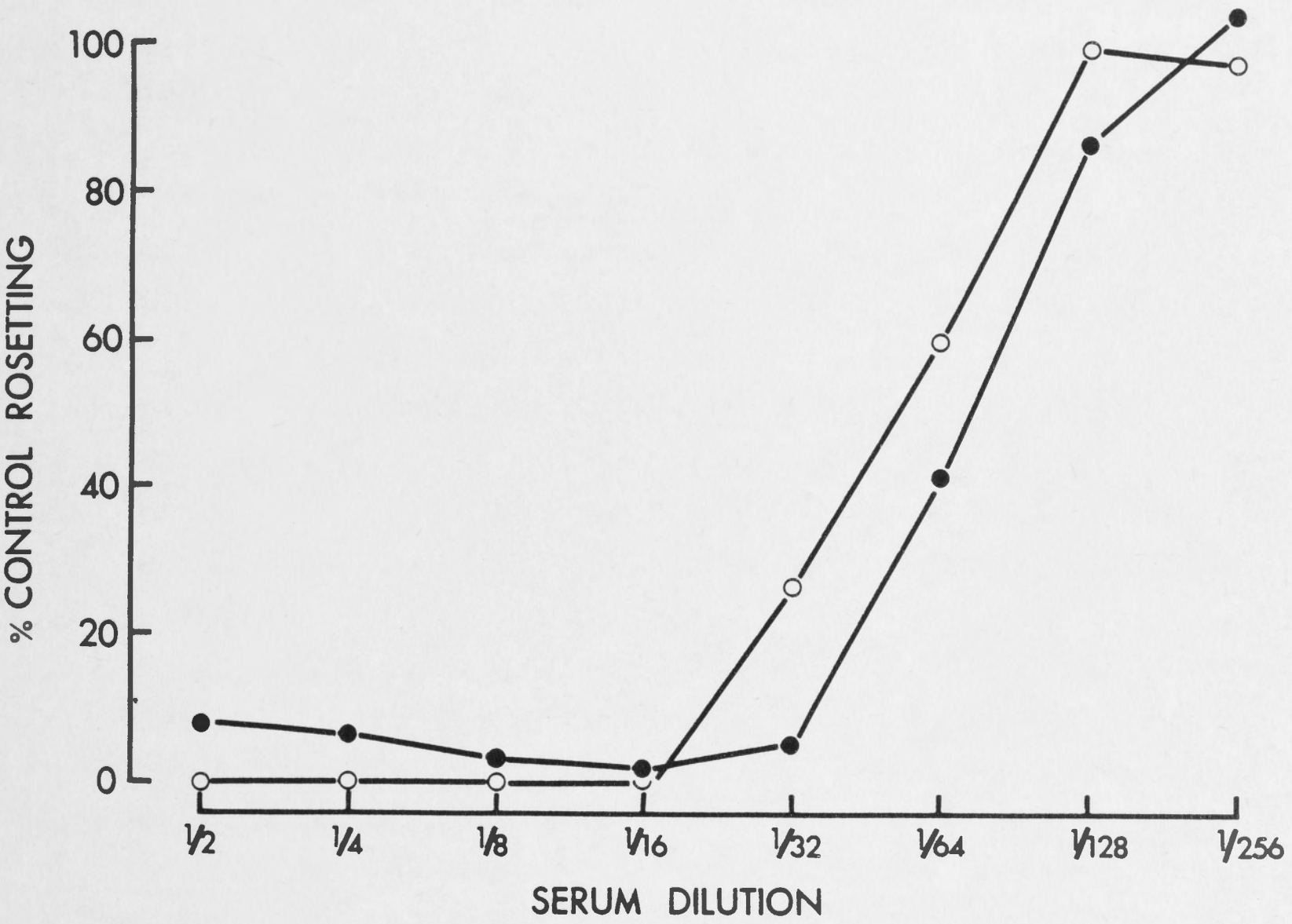


Fig. 2. Inhibition of autorosetting of BALB/c thymocytes by serial dilutions of BALB/c serum as detected by microscopic assay (○) or by Coulter Counter assay (●).

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